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Effect of Retinal Extracts on Growth of Blinded Male Rats.*

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It is known that bilateral optic enucleation delays the onset of sexual maturity of both male and female rats.[1,2,3] Bilateral optic enucleation also causes the body weights and organ weights of blinded animals to be significantly less than those of litter-mate normal males, regardless of the environmental light conditions.[2,4] Fiske[5] reported that onset of sexual maturity under conditions of constant dark was delayed in normal animals by approximately 2 weeks, and that normal males in constant dark had lighter testicles, seminal vesicles, and pituitaries than did males in continuous light.

The evidence is quite consistent that total ablation of both eyes retards growth and sexual maturity in the rat. It is possible that the eye exerts its influence on the pituitary which in turn produces growth and gonadotropic substances. The interaction between the eye and the pituitary may be either via direct or indirect nervous connections between the eye and the pituitary, or via a humoral mechanism. Truscott[6] has described nervous pathways which might form the means by which visual stimuli could influence the anterior pituitary. The present investigation, however, is based on the hypothesis that some "principle" or humoral mechanism is elaborated by the eyes of normal mammals which is necessary for normal growth and sexual maturity.

A series of crude extracts of light-adapted beef retinas was prepared and injected into an inbred strain of blinded male rats. Eyes of freshly butchered cattle were removed and immediately placed in chilled sterile physiological saline by a veterinarian or by the author. The retinas were then dissected out under sterile saline and placed in appropriate media. All extracts of beef eyes, other preparations, and injections were carried out by the junior author, while all autopsies of experimental animals were performed by the senior author. The senior author had no

* This investigation was supported by grant from the Committee for Research in the Problems of Sex, National Research Council.

1 Browman, L. G., *Anat. Rec.*, 1938, **72**, 122.

2 Browman, L. G., *Anat. Rec.*, 1940, **78**, 59.

3 Truscott, B. L., *J. Exp. Zool.*, 1944, **95**, 291.

4 Browman, L. G., *Anat. Rec.*, 1938, **72**, 41.

5 Fiske, V. M., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 189.

6 Truscott, B. L., *J. Comp. Neurol.*, 1944, **80**, 2.

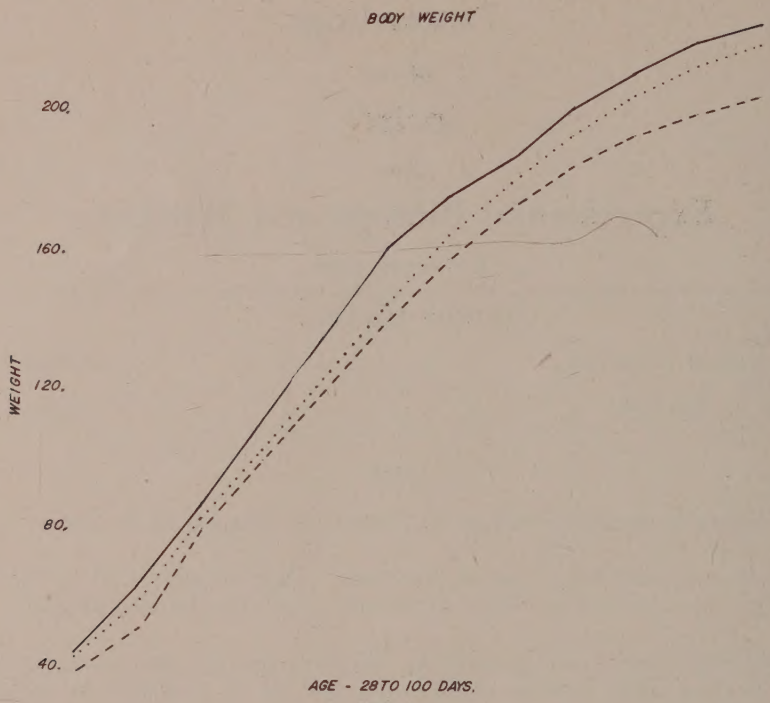

FIG. 1.

Average weekly body weights of
———— 20 normal eyed males.
– – – – – – 20 bilateral optic enucleated males.
. . . . . 10 bilateral optic enucleated males injected with saline extract of beef eyes.

knowledge as to which experimental series any particular animal belonged in until after autopsy.

Since only the saline series yielded suggestive results no space will be taken in discussion of the methods, results, etc., concerning ether fraction extracts, posterior lobe powder (melanophore) preparations, beta carotene solutions, and other preparations.

A series of 40 male rats equally divided between normal-eyed and bilateral optic enucleated on day one were autopsied on day 100. Inspection and statistical analysis of the data show that the blind animals had significantly lighter body, testis, seminal vesicle, and pituitary weights than litter-mate normal animals, although the thyroid glands of blinded animals were heavier than those of normal animals (Fig. 1, Table I).

Five beef retinas were macerated in 4 cc of Locke's mammalian saline by shaking vigorously with sand for 5 minutes. This mixture was leached overnight at 2°C and filtered by suction. This extract was prepared and injected 3 times weekly. It was calculated that each rat received a dose equivalent to an extract of 7.3 rat retinas by weight at each injection, or a daily average of 3 rat retinas. These injections began when the rats were 15 days of age and continued for 10 weeks. All animals were autopsied at 100 days of age. Another series of males receiving similar saline extracts was autopsied at intervals of 5 days beginning at 30 days of age, and the last pair in this series was autopsied at 70 days of age.

The number of animals involved in each injection series is not as large as was originally planned because of circumstances beyond our control. The differences between each experimental series are not always sharp and clear cut in magnitude, yet the results are

TABLE I.
Average Weights and Standard Errors at 100 Days of Age.

Injection	Cond. of eye	No. of animals	Body, g	Testes, g	Sem. V., mg	Adr., mg	Thym., mg	Thyr., mg	Pit., mg
None	Nor.	20	224 ± 4	3.0	681 ± 91	34.2	257 ± 9	13.2 ± .3	9.2 ± .2
"	Bl.	20	207 ± 3	2.8	511 ± 78	34.2	261 ± 9	14.8 ± .4	7.4 ± .2
Saline Ext.	Bl.	10	222 ± 5	2.9	735 ± 56	34.0	238 ± 16	12.7 ± .9	7.6 ± .4
B. Carotene	Bl.	10	206 ± 1	2.8	496 ± 24	32.8	269 ± 9	15.0 ± .5	8.2 ± .2
Post. Lobe	Bl.	10	202 ± 1	2.8	504 ± 54	32.2	261 ± 8	14.2 ± .5	7.1 ± .1
Ether Extr.	Bl.	9	190 ± 4	2.5	515 ± 33	29.8	271 ± 17	15.8 ± .5	7.8 ± .1

sufficiently consistent to warrant this report.

Reference to Table I indicates that at 100 days of age the males from the saline extract series more nearly approached the body and organ weights of normal males of the same age than did the animals in any other experimental group. This is especially true of the testis, seminal vesicle, and thyroid weights. Fig. 1 indicates that the males which received saline extracts averaged consistently heavier than bilateral enucleated controls. Injections of sterile saline only into blinded males had no alleviating influence on weight curves.

Five-day Series. One male of each pair autopsied at 5-day intervals received the saline extract beginning day 12, while the blinded litter-mate male received none. Litter-mate pairs were contrasted and revealed: (1) that injected males were heavier in body, testis, seminal vesicle, adrenal, liver, thyroid, and pituitary weights up to age 70 days, (2) that there was no marked difference in thymus weights, (3) that spermatozoa were present in the testes of injected animals by 45 days, and in non-injected males by 50 days. Spermatozoa are present in normal animals of this strain by 35-40 days of age. (4) Although seminal vesicle weights were essentially the same up to day 45, by 65 days, the seminal vesicles of non-injected males were only 10 times, injected animals were 18 times, and normal animals were 20 times the 45-day weight.

Summary. Normal male rats, and bilateral optic enucleated male rats injected with a saline extract of bovine retinas are quite similar in body and most organ weights. Retinal extracts of bovine eyes apparently have an alleviating effect upon the weight retardation and delayed sexual maturity characteristic of optic enucleated male rats.

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Studies on Purification of Poliomyelitis Virus.* 2. pH Stability Range of MVA Strain.

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While it is known that poliomyelitis virus activity is preserved for long periods of time in infected tissues[1] and in sewage,[2] little has been published regarding its stability under conditions where the virus is exposed to the more extreme hydrogen ion concentrations.

* Aided by a grant from The National Foundation for Infantile Paralysis, Inc.

1 Flexner, S., and Lewis, P. A., *J. Am. Med. Assn.*, 1910, **54**, 45; Römer, P. H., and Joseph, K., *Münch. med. Woch.*, 1910, **57**, 347; Flexner, S., Clark, P. F., and Amoss, H. L., *J. Exp. Med.*, 1914, **19**, 205; Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1917, **25**, 539; Rhoads, C. P., *J. Exp. Med.*, 1929, **49**, 701.

2 Paul, J. R., and Trask, J. D., *J. Am. Med. Assn.*, 1941, **116**, 493.

The experiments of Sabin[3] on the extraction of virus with dibasic phosphate and those of Amoss[4] suggest a stability to solutions as alkaline as pH 8 or 9. Howitt[5] has found virus activity after exposure to pH 4.4 and heating to 58°, and more recently Schultz and Robinson[6] have reported that the virus survives exposure to pH 2.2 in the presence of cysteine and to pH 10.4 in the presence of sodium peroxide. Treatment with a stronger sodium peroxide solution at pH 11.4, however, caused a loss of virus activity.

The above mentioned experiments suggest that poliomyelitis virus possesses an unusual stability to the extreme ranges of hydrogen ion concentrations. As such experiments have been carried out on relatively crude virus extracts, however, it is not certain that virus freed from the various tissue components would show the same behavior. Additional information of this nature would be of value not only in providing further characterization of the virus and in showing the relationship between the various strains but might also prove useful for purification purposes. In the present paper experiments are presented in which the stability of the MVA strain[7] was studied over the acid range from pH 1 to 4 and over the alkaline range from pH 8 to 11.

Experimental. The virus preparations used in every case consisted of samples that had been purified by ultracentrifugation.[8] It has been shown that such preparations produce poliomyelitis in rhesus monkeys in from 50 to 100% of the animals injected intracerebrally with 5×10^{-9} g of purified virus nitrogen. This amount was, therefore, estimated to contain between 1 and 10 minimal infective doses. The method used to determine whether or not virus activity was destroyed by a given hydrogen ion concentration was to

TABLE I. Summary of pH Stability Experiments.

Exp. No.	Conc. in mg N per ml	Control in Ringer's	pH range ± 0.49									
			1	2	3	4	7	8	9	10	11	
26	5×10^{-8}	+ + + *			—	+ —	+ —	+ —	+ + + —	+ (10.0) †		
	5×10^{-7}	+ + +			+ +	+	+ +	+ + + —				
34	5×10^{-9}	+ +		+ — (2.4)		+ +		+ —	+ + + —			
35	5×10^{-9}	+ + —		+ + + (1.6)	+ + —		+ +		+ —	+ + (10.1)	— — (11.2)	
				+ + — — (2.0)								
36	5×10^{-9}	+ —	— (1.0)	+ — (1.6)	+ —				— —	+ (10.0)		
	5×10^{-8}		— — (1.0)								— — (11.0)	
	5×10^{-7}										+ — (11.0)	
38	5×10^{-9}	+ —	— (1.0)	+ — (1.6)	+ —				+ —	+ — (10.3)	— — (10.9)	
	5×10^{-8}		— — (1.1)	+ — (1.6)					+ —	+ — (9.8)	— — (11.0)	

* Each + sign indicates that the monkey developed poliomyelitis.
Each — sign indicates that the monkey failed to develop poliomyelitis within 3 weeks, but later either proved susceptible or died from other causes.
† The numbers in parentheses are the actual pH values for the more extreme hydrogen ion concentrations.

[3] Sabin, A. B., *J. Exp. Med.*, 1932, **56**, 307.

[4] Amoss, H. L., in Rivers' *Filterable Viruses*, 1928, p. 159.

[5] Howitt, B. F., PROC. SOC. EXP. BIOL. AND MED., 1930, **28**, 158.

[6] Schultz, E. W., and Robinson, F., *J. Inf. Dis.*, 1942, **70**, 193.

[7] Schultz, E. W., and Gebhardt, L. P., *J. Inf. Dis.*, 1942, **70**, 7.

[8] Loring, H. S., and Schwerdt, C. E., *J. Exp. Med.*, 1942, **75**, 395.

find if this same amount of virus would produce poliomyelitis after it had been exposed to the desired pH and the solution readjusted to neutrality. If the solution proved completely inactive after this treatment, it is evident that less than one MID of active virus remained and that the pH in question destroyed most of the virus activity. If the solution proved infectious for some of the animals, the only conclusion that could be drawn was that an appreciable amount of virus activity withstood the treatment. Although an untreated control of the same concentration as the test solution was also injected in most of the experiments, the number of animals used was not sufficiently large to provide a more exact description of the amount of virus which survived the treatment. In cases where no virus activity was found with a concentration of 5×10^{-9} g after exposure to a given pH, the amount of virus was increased ten or a hundredfold, and the activity of these solutions determined after exposure to the same pH. In this way a rough measure was obtained of the amount of virus above the range of 1 to 10 MID destroyed by the treatment.

Phosphate-lactate buffers were employed for the initial pH stability experiments. Their buffering capacities were found too limited at the extreme pH values, however, consequently Michaelis' veronal-acetate-hydrochloric acid buffer[9] was used for solutions at pH 1 to 9, and Sorensen's borate-sodium hydroxide buffer[10] for the solutions between pH 10 and 11. Both buffer solutions were sterilized by autoclaving.

Purified virus preparations were exposed to the various buffers as follows: 0.5 ml of virus in Ringer's solution, one-tenth the concentration of that desired for the final inoculum, was mixed with 3.0 ml of buffer and the mixture allowed to stand at room temperature for 15 to 20 minutes. One ml of this solution was used to determine the exact pH by means of a glass electrode. After the allotted time for exposure had elapsed, the remaining 2.5 ml were approximately neutralized with 0.1 N or 0.5 N hydrochloric acid or sodium hydroxide. A previous electrometric titration of the buffer employed facilitated the estimation of standard acid or alkali required to adjust the hydrogen ion concentration to neutrality. Finally the calculated amount of sterile distilled water was added to effect a final dilution of the virus suspension ten times that of the original preparation exposed to the buffer.

Within one to 2 hours after exposure to the desired pH and readjustment, the neutralized virus-buffer mixture was inoculated intracerebrally into rhesus monkeys. One ml of virus solution was used for each inoculation.

The results obtained with 5 different purified preparations after exposure to the various hydrogen ion concentrations are summarized in Table I. It may be seen from the control in which the same amount of virus in Ringer's solution was inoculated as in the test solutions that about 50% of the animals inoculated with a concentration of 5×10^{-9} g of purified virus nitrogen developed poliomyelitis. Similar results were found for solutions which had been exposed to pH 1.6 or to pH 10 $\pm$ 0.3 or to the various hydrogen ion concentrations tested between these values. Examination of the table also shows that in no case was the solution infectious after exposure to pH 1.0 or to pH 11 $\pm$ 0.2 when tested at either a concentration of 5×10^{-9} or 5×10^{-8} g of purified virus nitrogen. In one experiment in which the virus concentration injected after exposure to pH 11 was increased to 5×10^{-7} g, however, one of two animals injected developed poliomyelitis. It would appear therefore that about 1% of active virus remained in this case.

Summary. Experiments are presented in which the stability of the MVA strain of poliomyelitis virus to acid and alkaline solutions has been determined. It is concluded that this strain when in purified form is relatively stable in solutions as acid as pH 1.6 or as alkaline as pH 10 $\pm$ 0.3 but is unstable in solutions more acid than the former or more alkaline than the latter values.

[9] Michaelis, L., *Biochem. Z.*, 1931, **234**, 139.

[10] Clark, W. M., *The Determination of Hydrogen Ions*, The Williams and Wilkins Co., Baltimore, 1928.

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Complement Fixation Antigens of Influenza Viruses Type A and B.*

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High speed centrifugation of allantoic fluid infected with the virus of influenza A and B at speeds sufficient to sediment almost completely the agent as contained in infected mouse lungs frequently leaves a more or less substantial portion of the virus in the supernate.[1,2] This has suggested that the elementary infectious unit of the virus of influenza was distinctly smaller (about 10 mμ in diameter) than was thought formerly (80-100 mμ[3,4]). This concept seemed to receive support when analytical centrifugation revealed the presence in infected allantoic fluid of a small component with a sedimentation constant of approximately 30 S in addition to material sedimenting at a faster rate (sedimentation constant 800 ± 100 S). Electron-microscopy confirmed this finding.[5]

Recent studies of the influence of convection on sedimentation in the angle centrifuge showed that complete settling of suspended particles could not be atttained unless convection was counteracted.[6] The addition of a synthetic density gradient to influenza virus preparations was found to prevent resuspension of the agent by convection and it was shown that practically all of the infectivity and hemagglutinating property was linked to the larger particles.[7,8] This fraction on more extended studies by various workers was found to possess a sedimentation constant of between 600 and 800 S[2,7,8,9] and will be referred to hereafter as 600 S component. It was prepared from infected allantoic fluid by 2 cycles of alternate centrifugation at 20,000 and 3,000 r.p.m. for 20 minutes each.

The smaller material (30 S component†) obtained from allantoic fluid after removal of the 600 S component by alternate centrifugation at 30,000 and 20,000 r.p.m. for 60 and 20 minutes, respectively, failed to produce measurable hemagglutination, and possessed only a fraction of the infectivity of the 600 S component as shown by Stanley.[8] Additional purification by centrifugation decreased the infectivity further and it appeared likely that this property in preparations of the 30 S material was due to contamination by small amounts of the larger particles.[8] However, it was obvious that most of the 30 S material in the allantoic fluid resulted from infection with the influenza virus since it was found in high concentrations in cultures of recently isolated strains (F-12 and F-99 of influenza A), in smaller quantity in those of the Lee strain of influenza B or the PR-8 strain of influenza A virus, while very little

* This study has been aided by a grant from the Medical Research Division of Sharp and Dohme, Inc.

1 Chambers, L. A., and Henle, W., *J. Exp. Med.*, 1943, **77**, 251.

2 Taylor, A. R., Sharp, D. G., Beard, D., Beard, J. W., Dingle, J. H., and Feller, A. E., *J. Immunol.*, 1943, **47**, 261.

3 Elford, W. J., Andrewes, C. H., and Tang, F. F., *Brit. J. Exp. Path.*, 1936, **17**, 51.

4 Elford, W. J., and Andrewes, C. H., *Brit. J. Exp. Path.*, 1936, **17**, 422.

5 Chambers, L. A., Henle, W., Lauffer, M. A., and Anderson, T. F., *J. Exp. Med.*, 1943, **77**, 265.

6 Pickels, E. G., *J. Gen. Physiol.*, 1943, **26**, 341.

7 Friedewald, W. F., and Pickels, E. G., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 261; *J. Exp. Med.*, 1944, **79**, 301.

8 Stanley, W. M., *J. Exp. Med.*, 1944, **79**, 267.

9 Sharp, D. G., Taylor, A. R., McLean, I. W., Jr., Beard, D., Beard, J. W., Feller, A. E., and Dingle, J. H., *J. Immunol.*, 1944, **48**, 129.

† The material sedimentable from allantoic fluid at 90,000 g for 60 minutes after previous removal of the 600 S component has been called 30 S component for the sake of brevity although we are aware that it may be a mixture of a number of different materials (*cf.* Knight[10]).

10 Knight, C. A., *J. Exp. Med.*, 1944, **80**, 83.

material was sedimented from normal allantoic fluid of 10- to 13-day embryos under similar conditions.

Further information was gained as to the nature of these components by using the complement fixation test. The technic used has been described previously.[11] Both the 600 and 30 S components were serologically active *in vitro* and in addition the supernate of infected allantoic fluid after removal of these 2 components still contained some antigen which could not be sedimented by repeated centrifugation at 30,000 r.p.m.,[11] or by centrifugation in the presence of synthetic density gradients. This antigen will be called <30 S component. All human sera used in these tests were free of antibodies against Forssman antigen and chick proteins and they were Wassermann-negative.

The complement fixation tests showed that the reactions with the 600 and 30 S components from infectious allantoic fluids were specific. The tests were positive in the presence of human influenza convalescent serum but not with serum taken before or during the acute stage of the disease. Furthermore, the reactions were type-specific in that the 600 and 30 S components derived from influenza A virus preparations reacted only with influenza A and not with influenza B convalescent serum and conversely, the corresponding component prepared from Lee cultures gave positive tests only with influenza B and not influenza A convalescent serum. Evidence to support this statement is included in Table II. Some preparations of the 600 and 30 S components were kindly supplied by Dr. W. M. Stanley. These had been studied in the analytical centrifuge and had shown single boundaries.

The serological activity of the 30 S material could not be due to contamination of these preparations by small amounts of the 600 S component since comparison of the reactivities of the 600 and 30 S fractions on a N-basis revealed similarly strong serological potencies per mg of protein for both preparations. Further purification of the 30 S material by repeated centrifugation at 20,000 r.p.m. for 20-30 minutes, which removed small additional amounts of the 600 S component from suspension did not change the results of the complement fixation test. Furthermore, attempts to adsorb the antigen from 30 S preparations onto chick red cells failed while the 600 S component could be removed from suspension by this technic as shown by complement fixation as well as hemagglutination tests.

These experiments clearly demonstrated physical differences between the 2 antigen fractions. In addition, striking serological differences were noted between the 600 and 30 S components when their optimal antigen-antibody relationships were compared. An experiment with these preparations derived from the Lee strain of influenza B, summarized in Table I, serves to demonstrate this point. Furthermore, absorption of human convalescent sera with the 600 S component on the one hand, and the 30 or <30 S material on the other, clearly showed the occurrence of two distinct antigens in influenza virus preparations. When the sera were absorbed with small quantities of the 600 S component they no longer reacted with up to 32 units of this antigen while the reaction with an aliquot of the original allantoic fluid from which the absorbing material had been prepared, as well as with the homologous 30 S component, was only slightly changed. However, when high concentrations of the 600 S antigen (more than 32 units) were added to the absorbed serum positive results were obtained and the optimal antigen-antibody relationship was similar to that found with the 30 S fraction (*cf.* Table I). In agreement with this observation absorption of the convalescent sera with large amounts of the 600 S component removed all antibodies to the large particles as well as to the 30 S material. On the other hand, absorption of the convalescent sera with the $(NH_4)_2SO_4$ precipitate (<30 S) of the supernate of allantoic fluid after sedimentation of the 600 S and 30 S components removed all antibodies to the <30 S material and to the 30 S component but left a strong reaction with the 600 S material and the original allantoic fluid. These relations are demonstrated in Table II. Control tests

[11] Henle, W., Henle, G., Groupé, V., and Chambers, L. A., *J. Immunol.*, 1944, **48**, 163.

TABLE I.
Optimal Antigen-Antibody Relationships Encountered with 600 and 30 S Components.

Antigen	Serum dilution	Antigen dilution									
		und.	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512
600 S	1: 8	0	0	0	0	0	0	0	0	wk	c
LEE	1: 16	0	0	0	0	0	0	0	tr	ac	c
	1: 32	0	0	0	0	0	0	tr	st	c	c
	1: 64	0	0	0	0	0	tr	ac	c	c	c
	1:128	st	tr	0	tr	wk	c	c	c	c	c
	1:256	c	ac	ac	c	c	c	c	c	c	c
	1:512	c	c	c	c	c	c	c	c	c	c
30 S	1: 16	0	0	0	0	st	st	c			
LEE	1: 32	0	0	0	0	st	ac	c			
	1: 64	0	0	0	0	ac	c	c			
	1:128	wk	tr	0	0	ac	c	c			
	1:256	c	ac	st	st	ac	c	c			
	1:512	c	c	c	c	c	c	c			

0—no hemolysis; tr—trace; wk—weak; st—strong; ac—almost complete; c—complete hemolysis.

showed that absorption of an influenza A convalescent serum with the influenza B fractions did not affect the homologous reactions and vice versa. The inhibition of red cell agglutination and neutralization of mouse infectivity by the sera after absorption with the 600 S component was negative, while absorption with the <30 S material altered only slightly the results as compared with the control tests using the unabsorbed sera.

The finding of 2 serologically distinct antigens in allantoic fluids infected with influenza A or B is in agreement with former observations.[11] It had been noted before that material sedimentable at 90,000 g in 1 hour (containing both the 600 S and 30 S components) possessed 2 distinct antigens while the supernate contained only one which was similar to one of the sedimentable antigens. Friedewald[12] noted recently that the "soluble antigen" of influenza showed different properties in many respects from the antigen associated with the virus. The relation between the 30 S component and the 600 S material requires further investigation. In view of the above absorption experiments the 30 S material probably constitutes only a small fraction of the 600 S particle but may be continuously released from it into the surrounding medium; or the 30 S material may not be exposed at the surface of the large component to any great extent but is freed only upon its disintegration. In this regard it is of interest to note that vibration of the 600 S component in the treatment vessel of a magnetostriction oscillator[13] at 90,000 cycles per second released some antigen from the large component which reacted serologically like the 30 S antigen.

Since sera of human convalescents from experimental infection with 2 distinct strains of influenza A virus (PR-8 and F-99) were available the strain specificity of the various components was studied by serum absorption tests. No difference was noted between the 30 S components of the PR-8 and F-99 strains, but the 600 S materials yielded strain-specific complement fixation tests in addition to a common antigenic activity. As shown in Table III, absorption of convalescent sera with the 600 S component of the homologous virus removed the complement-fixing antibodies to both the homologous and heterologous antigens, whereas the heterologous 600 S material absorbed only the heterologous antibodies but left some activity with the homologous 600 S antigen. Strain-specific dominance of certain immune or convalescent sera in the complement fixation test had been noted previously.[12,14,15] Similar results of

[12] Friedewald, W. F., *J. Exp. Med.*, 1943, **78**, 347.

[13] Chambers, L. A., and Gaines, N., *J. Cell. and Comp. Physiol.*, 1932, **1**, 451.

[14] Lush, D., and Burnet, F. M., *Austral. J. Exp. Biol. and Med. Sc.*, 1937, **15**, 375.

[15] Eaton, M. D., *J. Immunol.*, 1941, **41**, 383.

TABLE II.
Demonstration of 2 Antigenic Components in Allantoic Fluid Infected with Influenza A or B Virus.

Antigen Strain fraction	Optimal titer of serum									
	Convalescent influenza A absorbed with					Convalescent influenza B absorbed with				
	—	PR-8 600S	PR-8 <30S	Lee 600S	Lee <30S	—	PR-8 600S	PR-8 <30S	Lee 600S	Lee <30S
PR-8 Orig. all. fl.	1:32	1:16	1:16	1:32	1:32	1:4	<1:4	<1:4	1:4	1:4
600S	1:32	<1:4	1:16	1:32	1:32	1:4	<1:4	<1:4	1:4	1:4
30S	1:16	1:16	<1:4	1:16	1:16	<1:4				
<30S	1:8	1:8	<1:4	1:8	1:8	<1:4				
Lee Orig. all. fl.	<1:4					1:64	1:64	1:64	1:32	1:32
600S	<1:4					1:32	1:32	1:32	1:4	1:32
30S	<1:4					1:64	1:64	1:64	1:32	1:8

1:32 = highest initial serum dilution giving complete fixation of complement in the presence of from .5-16 units of antigen. All serum and antigen controls were completely hemolyzed.

TABLE III.
Strain-Specificity of Fractions Derived from 2 Strains of Influenza A Virus.

Antigen Strain fraction	Optimal Titer of serum									
	Convalescent PR-8 absorbed with					Convalescent F-99 absorbed with				
		600S		<30S			600S		<30S	
	—	PR-8	F-99	PR-8	F-99	—	PR-8	F-99	PR-8	F-99
PR-8 Orig. all. fl.	1:32	1:16	1:16	1:16	1:16	1:64	1:32	1:32	1:16	1:32
600S	1:32	<1:4	*1:16*	1:16	1:32	1:32	<1:4	<1:4	1:8	1:32
30S	1:16	1:16	1:16	<1:4	<1:4	1:64	1:64	1:64	<1:4	<1:4
F-99 Orig. all. fl.	1:32	1:16	1:16	1:8	1:16	1:128	1:64	1:64	1:32	1:32
600S	1:16	<1:4	<1:4	1:8	1:8	1:64	*1:8*	1:4	1:16	1:32
30S	1:32	1:32	1:16	<1:4	<1:4	1:128	1:128	1:64	<1:4	<1:4

1:32 = highest initial serum dilution giving complete fixation in the presence of from 0.5-16 units of antigen. All serum and antigen controls were completely hemolyzed.

absorption tests were reported recently by Friedewald.[16]

Summary. Allantoic fluids infected with influenza A or B virus contain 3 antigenic fractions as measured by complement fixation technic: 1. sedimentable at relatively low speeds and directly linked with virus activity (600 S); 2. sedimentable at 90,000 g (30 S) possibly identical with the antigen observed in mouse lungs by Lennette and Horsfall;[17] 3. non-sedimentable at 90,000 g upon repeated centrifugation or in the presence of a sucrose density gradient (<30 S). The serological activity of fraction 2 and 3 appears to be based on one antigen, while the activity of fraction 1 is mainly due to another antigen distinct from fractions 2 and 3, as shown by serum absorption technic. The 30 S components of 2 strains of influenza A virus were found indistinguishable by serum absorption technic while the 600 S components revealed strain-specificity in addition to antigenic activity common to both strains.

[16] Friedewald, W. F., *J. Exp. Med.*, 1944, **79**, 633.

[17] Lennette, E. H., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1940, **72**, 233.

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The *In Vitro* Effects upon Sulfonamides of Local Anesthetics Derived from N-Substituted *p*-Aminobenzoic Acids.*

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Following the discovery by Woods[1] that the antibacterial effects of the sulfonamides could be blocked by small amounts of *p*-aminobenzoic acid, it was found that diethylaminoethyl *p*-aminobenzoate hydrochloride (procaine hydrochloride, U. S. P.) and other local anesthetics which are esters of *p*-aminobenzoic acid also have antisulfonamide action.[2] Peterson and Finland[3] recently demonstrated that procaine, in amounts ordinarily employed for local anesthesia, may be absorbed into the circulation in sufficient concentration to exert a definite inhibitory effect on the action of sulfonamide drugs that may be present in the blood. They furthermore noted that in spite of the presence in the body of bacteriostatic concentrations of sulfonamides, an infection may become locally established in an area which has been infiltrated with procaine.

In studies with hemolytic streptococcal infections in mice, De Waal *et al.*[4] observed that the antagonistic effect of procaine against sulfanilamide is most marked when large doses are given early in the course of sulfanilamide treatment. They noted, moreover, that although repeated small doses of the anesthetic reduced the efficacy of sulfanilamide therapy, occasional small doses had no antagonistic effect and occasional large doses had no lasting antagonistic effect. This may possibly be explained by Legge and Durie's[5] observation that in the mouse injected procaine is hydrolyzed to *p*-aminobenzoic acid, some of which is then acetylated to *p*-acetylaminobenzoic acid. Acetylation of the amino group of *p*-aminobenzoic acid leads to a 10,000-fold decrease in its antisulfonamide activity.[1]

On the basis of these findings it appeared worth while to determine whether a similar substitution of an acetyl group on the aromatic nitrogen of procaine would correspondingly affect the antisulfonamide action of the local anesthetic. In the course of these investigations derivatives of procaine were studied in which substitutions of alkyl groups were also made in the position mentioned. Dimethylaminoethyl *p*-butylaminobenzoate hydrochloride (tetracaine hydrochloride, U. S. P.), an anesthetic derived from *p*-butylaminobenzoic acid, and the acetyl derivative of α,β-dimethyl-γ-dimethylaminopropyl *p*-aminobenzoate hydrochloride (butamin, N. N. R.) were included among the compounds tested for antisulfonamide action.

Methods and Results. 1. *Tests in Nutrient Broth Medium.* A series of dilutions of the local anesthetics and their derivatives ranging from 1:100 to 1:1,000,000 were prepared in distilled water. 1.0 cc of each dilution was added to 8.7 cc of veal-infusion-dextrose broth containing 20 mg % of one of the sulfonamides. The tubes were autoclaved at 10 pounds for 10 min., and upon cooling 0.1 cc normal horse serum was added to each, followed by 0.2 cc of a 1:1,000 dilution of a 24-hour broth culture of the β-hemolytic streptococcus C-203. This gave a final series of dilutions of the local anesthetics ranging from 1:1,000 to 1:10,000,000. The tubes were incubated at 37°C and examined after 24 hours for visible growth. The results obtained with sulfanilamide, sulfathiazole, sulfadiazine, and sulfa-

* Presented in part at the 45th Annual Meeting of the Society of American Bacteriologists, New York City, May 3, 1944.

1 Woods, D. D., *Brit. J. Exp. Path.*, 1940, **21**, 74.

2 Keltch, A. K., Baker, L. A., Krahl, M. E., and Clowes, G. H. A., Proc. Soc. Exp. Biol. and Med., 1941, **47**, 533.

3 Peterson, O. L., and Finland, M., *Am. J. M. Sc.*, 1944, **207**, 166.

4 De Waal, H. L., Kanaar, A. C., and McNaughton, J., *Lancet*, 1942, **2**, 724.

5 Legge, J. W., and Durie, E. B., *M. J. Australia*, 1942, **2**, 561.

TABLE I.
Limiting Dilutions Affecting Sulfonamide Action Against Beta Hemolytic Streptococcus.

Anesthetic	Sulfonamide—20 mg %			
	Sulfanilamide	Sulfadiazine	Sulfathiazole	Sulfapyridine
Procaine hydrochloride—U.S.P.	$10^{-7}>$	10^{-6}	10^{-6}	10^{-6}
Procaine hydrochloride	10^{-5}	2×10^{-5}	10^{-3}	10^{-4}
Ethyl derivative				
Procaine hydrochloride	2×10^{-5}	10^{-4}	$<2\times10^{-4}$	$<2\times10^{-4}$
Butyl derivative				
Procaine hydrochloride	10^{-3}	$<10^{-3}$	$<10^{-3}$	$<10^{-3}$
Acetyl derivative				
Butamin, N. N. R.	10^{-5}	10^{-5}	2×10^{-4}	10^{-4}
Butamin	$<10^{-3}$	$<10^{-3}$	$<10^{-3}$	$<10^{-3}$
Acetyl derivative				
Tetracaine hydrochloride—U.S.P.	$<2\times10^{-4}$	$<2\times10^{-4}$	$<2\times10^{-4}$	$<2\times10^{-4}$

$10^{-7}>$ indicates highest dilution studied proved antagonistic to sulfanilamide; $<2\times10^{-4}$ indicates no antagonistic effect in this concentration, furthermore, the greater concentration (10^{-3}) proved to be bacteriostatic in presence or absence of sulfonamides; $<10^{-3}$ indicates greatest concentration studied had no antisulfonamide action.

TABLE II.
Effects of Local Anesthetics upon Sulfathiazole Activity Against *E. coli.*

Molar conc. of sulfathiazole	Molar Concentrations of Anesthetics									
	Procaine					Ethyl Procaine				
	10^{-3}	10^{-4}	10^{-5}	10^{-6}	none	10^{-3}	10^{-4}	10^{-5}	10^{-6}	none
10^{-3}	4	0	0	0	0	0	0	0	0	0
4×10^{-3}	4	4	1	0	0	0	0	0	0	0
1.6×10^{-4}	4	4	4	0	0	1	0	0	0	0
6.4×10^{-4}	4	4	4	4	4	3	2	1	1	1
None	4	4	4	4	4	4	4	4	4	4
	Butyl Procaine					Acetyl Procaine				
10^{-3}	0	0	0	0	0	0	0	0	0	0
4×10^{-3}	0	0	0	0	0	0	0	0	0	0
1.6×10^{-4}	0	0	0	0	0	0	0	0	0	0
6.4×10^{-4}	1	0	0	0	0	0	0	0	0	2
None	4	4	4	4	4	4	4	4	4	4
	Butamin					Acetyl Butamin				
10^{-3}	0	0	0	0	0	0	0	0	0	0
4×10^{-3}	2	0	0	0	0	0	0	0	0	0
1.6×10^{-4}	4	3	0	0	0	0	0	0	0	0
6.4×10^{-4}	4	4	4	0	2	0	0	0	0	0
None	4	4	4	4	4	4	4	4	4	4

The anesthetics were used in the form of the hydrochlorides. 0 = no antisulfathiazole activity; 1-3 = increasing degrees of antisulfathiazole activity; 4 = complete antisulfathiazole activity, or growth equivalent to control.

pyridine are given in Table I.

Results of tests in broth medium. From the data presented in the table it is apparent that procaine exhibits a high degree of antisulfonamide activity against all the sulfonamides tested. With the substitution of an ethyl group on the aromatic nitrogen, the antisulfonamide action of the local anesthetic is markedly decreased. A butyl group substituted in this position further decreases this effect, and with the substitution of an acetyl group, the antisulfonamide activity is practically eliminated. Butamin, which is also a derivative of *p*-aminobenzoic acid, has approximately half as much antisulfonamide activity as procaine. This may be due to the fact that the former is a substituted secondary alkyl ester which would be expected to hydrolyze more slowly to *p*-aminobenzoic acid than does procaine. Acetylation of butamin destroys its antagonistic reaction toward the sulfonamides. Tetracaine, the derivative of *p*-butylaminobenzoic acid, failed to give any indication of antisulfonamide action. Although not given

in the table, other local anesthetics which are not derivatives of *p*-aminobenzoic acid were also tested and found to have no effect upon sulfonamides. This group included N,N′-*p*-ethoxy phenylacetamidine hydrochloride monohydrate (phenacaine hydrochloride, U. S. P.), β-diethylaminoethylamide of 2-butoxy-cinchoninic acid hydrochloride 2-butoxy-4-(β-diethylaminoethylamido) carboxy quinoline hydrochloride (dibucaine, N. N. R.), and 2-benzoxy-2-dimethylaminomethyl-1-dimethylaminobutane hydrochloride (amydricaine, N. N. R.).

2. *Tests in Synthetic Medium.* In this series of tests the most active sulfonamide studied, sulfathiazole, was mixed in varying concentrations with a series of dilutions of the local anesthetics. A synthetic medium described by Kalmanson and Bronfenbrenner[6] was used with *Escherichia coli* as the test organism. A 10^{-2} molar concentration of each local anesthetic was prepared by dissolving the compound in distilled water. The solution was sterilized by boiling for 2 to 3 min., following which it was cooled rapidly to room temperature. Subsequent dilutions of 10^{-3}, 10^{-4}, and 10^{-5} molar concentrations were made aseptically from the initial dilution in sterile distilled water. In a similar manner a 10^{-2} molar concentration of sulfathiazole was prepared and then diluted further to give molar concentrations of 4×10^{-2}, 1.6×10^{-3}, and 6.4×10^{-3}.

Each dilution of an anesthetic was combined with each concentration of sulfathiazole by aseptically pipetting sterile solutions, per tube, as follows:

		cc
Synthetic medium		6.0
Local anesthetic solution		1.0
Sulfathiazole	"	1.0
5% dextrose	"	0.4
Distilled water		1.5
		9.9

This resulted in a final molar concentration of 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} of the anesthetic and 10^{-3}, 4×10^{-3}, 1.6×10^{-4}, and 6.4×10^{-4} of sulfathiazole. The inoculum consisted of a 0.1 cc of a 24-hour growth of *E. coli* in the synthetic medium, diluted 1:10 with the same medium. Controls were maintained by omitting in one instance the local anesthetic, in another sulfathiazole, and a final control lacking both these ingredients. All tubes were incubated at 37°C and examined after 48 hours. The results of these tests are given in Table II.

Results of tests in synthetic medium. The same general trend of antisulfonamide activity is again displayed by procaine and butamin as noted in the previous tests in broth medium. The efficacy of alkylation and acetylation of the anesthetics in reducing their antisulfathiazole activity is again apparent.

Discussion. The antagonistic action of *p*-aminobenzoic acid against the sulfonamides has been a subject of considerable interest during the past few years. It has recently been found that various esters of *p*-aminobenzoic acid exert this same antagonism in varying degrees. Among the esters of *p*-aminobenzoic acid are procaine, butamin, mono-*n*-amylaminoethyl-*p*-aminobenzoate hydrochloride (amylcaine hydrochloride, N. N. R.), γ-dibutylaminopropyl-*p*-aminobenzoate-N-sulfate (butacaine sulfate, U. S. P.), γ-diethylamino-β-β-dimethylpropyl-*p*-aminobenzoate hydrochloride (larocaine hydrochloride, N. N. R.), and *n*-butyl-*p*-aminobenzoate (normal butyl aminobenzoate, U. S. P.) Upon saponification of any of these anesthetics, the probable end-product is *p*-aminobenzoic acid.

Other local anesthetics which are not esters of *p*-aminobenzoic acid have failed to show evidence of any antisulfonamide activity. This latter group includes tetracaine, a derivative of *p*-butylaminobenzoic acid, which, contrary to our observations, was found by Keltch *et al.*[2] to have an antagonistic action to sulfonamides. This raised the question as to whether similar substitutions of alkyl groups in those anesthetics derived from *p*-aminobenzoic acid would also affect their antisulfonamide action. Furthermore, inasmuch as acetylation of *p*-aminobenzoic acid is known to markedly reduce its antagonism towards sulfonamides, it could be assumed that acetyla-

6 Kalmanson, G., and Bronfenbrenner, J., *J. Gen. Physiol.*, 1939, **23**, 201.

tion of its esters would produce the same result. With this thought in mind, the series of alkyl and acetyl derivatives of procaine and the acetyl analogue of butamin were prepared and studied.†

Our findings confirmed the above assumption in that the N-substituted anesthetics markedly decreased or neutralized completely the antisulfonamide action of these compounds.

Summary. Studies are presented which indicate that substitution of an alkyl group on the aromatic nitrogen of procaine will markedly reduce or inactivate completely the antisulfonamide action of the local anesthetic. Substitution of an acetyl group in the position mentioned on procaine, as well as butamin, will likewise result in an inactivation of the antisulfonamide property of these compounds.

† The authors wish to express their appreciation to Dr. A. R. Surrey for the preparation of these compounds and acknowledge the technical assistance of Virginia L. Wilson.

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Anomalous Offspring and Growth of Wistar Rats Maintained on a Deficient Diet.

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The nutritional deficiency of the maternal diet has been shown to influence the developmental anatomy of rats *in utero*. Gross congenital malformations were induced in approximately one-third of the offspring of rats of the Sprague-Dawley and Baltimore strains reared and maintained on a deficient diet.[1,2,3,4] However, Wistar rats similarly reared and maintained failed to produce anomalous young (of 21 Wistar female rats, few reached maturity, and only one female had a litter, consisting of one normal young.)[3] From these observations it was suggested that a strain difference exists in the reactivity of rats to the deficient diet. We have repeated the immature Wistar rat experiments of Warkany *et al.*[3] noted above, in an attempt to determine if Wistar rats might produce litters with either normal or abnormal offspring. In addition adult Wistar rats have been similarly maintained.

The data, presented below, indicate that adult Wistar rats maintained on the same diet can produce anomalous young and that immature Wistar rats reared and maintained on the same diet grow poorly and generally do not attain sexual maturity.

The experimental diet employed was the following: yellow corn meal, 76 parts; wheat gluten, 20 parts; calcium carbonate C. P., 3 parts; and iodized sodium chloride, 1 part.† At least 50 international units of vitamin D in cod liver oil were given each animal by pipette each week. This diet is definitely deficient in vitamins of the B_2 group and is probably deficient in inorganic elements and

* Present address: Department of Anatomy, Long Island College of Medicine, Brooklyn.

1 Warkany, J., and Nelson, R., *Anat. Rec.*, 1941, **79**, 83.

2 Warkany, J., and Nelson, R., *J. Nutrition*, 1942, **23**, 321.

3 Warkany, J., Nelson, R., and Schraffenberger, E., *Am. J. Dis. Child.*, **64**, 960.

4 Warkany, J., and Schraffenberger, E., *J. Nutrition*, 1944, **27**, 477. Riboflavin has been identified as the factor whose absence is responsible for the anomalous offspring.

† Diet is essentially the same as used by Warkany *et al.*[2,3,4] and Remington[5] with the exception that the former supplemented the diet with 60 international units of vitamin D as viosterol every tenth day and the latter supplemented the diet with .2% dried yeast.

FIG. 1. FIG. 2. FIG. 3.

The figures of littermate offspring of a Wistar rat maintained on the deficient diet. All specimens were cleared in potassium hydroxide and stained with alizarin red S.

FIG. 1. Normal offspring.

FIG. 2. Anomalous offspring with some skeletal abnormalities consisting of short mandibles and nubbin-like left tibia. This specimen is smaller than the normal offspring in Fig. 1.

FIG. 3. Anomalous offspring with pronounced anatomical malformations (description in text). Left upper extremity removed.

in the quality of proteins.[5]

Ten adult (ranging from 178 to 273 g) and twenty-one immature (ranging from 43 to 69 g) female Wistar rats were placed on the experimental diet. The breeding males and the 4 adult and 6 immature female control rats, all littermates to the experimental animals, were reared and maintained on the standard dog chow diet supplemented with greens. Diets were administered *ad libitum*.

Results. Gross malformations in the offspring of Wistar rats maintained on the deficient diet. The 10 adult experimental Wistar rats maintained on the deficient diet delivered, either normally or by caesarean operation, 17 litters of 67 offspring. Of these 67 progeny, 40 were normal, 23 (36.5% of 63 offspring) exhibited abnormalities and 4 were not classified as they were partially eaten by the mother.

The externally identifiable abnormalities were receding lower jaw, protruding tongue, short limb segments, and varying degrees of syndactylism of the fore and hind feet. Only 12 of the malformed young showed external abnormalities.

The osseous malformations‡ included the shortening of the mandible and premaxillary bone, varying degrees of shortening to com-

[5] Remington, R., *J. Nutrition*, 1937, **13**, 223.

‡ Skeleton observed in KOH cleared and alizarin red S stained specimens according to method of Noback, C., and Noback, E., *Stain Tech.*, 1944, **19**, 51.

plete absence of the radius, ulna, femur, tibia, fibula, and the bones of the fore and hind feet, the fusion of the ribs and various deformities of the sternum, humerus, maxilla, and scapula. All 23 malformed young showed one or more but never all of these malformations. Except for some minor quantitative differences, the external and osseous malformations observed in these animals are essentially the same as described by Warkany and Nelson[1] for Sprague-Dawley rats.

Two rats had distinctive anomalies. One specimen had an umbilical hernia but it was otherwise normal. The other specimen (Fig. 3), more deformed than any described by Warkany and Nelson,[1,2,3,4] had a normal skull, upper extremities and cervical, thoracic and lumbar vertebræ. However its ribs were short, and inclined caudally, making acute angles with the vertebral column, while the osseous elements of the sacral and caudal vertebræ and of both lower extremities, including the pelvis, were absent. No demonstrable resorption was noted. Of 50 control offspring prepared for osseous examination, all were normal.

The growth and reproduction of immature rats on the deficient diet. Of the 21 immature Wistar rats reared on the deficient diet, 19 did not attain adult size or mate and, after gaining from 18 to 60 g, they regressed in weight. The remaining 2 of the 21 rats attained weights of 185 and 167 g and mated. Of these 2 rats, one completely resorbed her one litter while the other gave birth to one abnormal and 2 normal young and probably resorbed 5 fetuses (indicated by 8 implantation sights at autopsy).

Similar difficulty in rearing immature Wistar rats to sexual maturity is reported by Warkany *et al.*[3] and Remington.§ However, immature female rats of either the Sprague-Dawley strain or Baltimore strain can be reared on this diet to attain sexual maturity and to produce litters with anomalous young.[1,2,3] On the basis of this difference between Sprague-Dawley and Baltimore strain rats and Wistar strain rats, a strain difference was suggested. As our results show that adult Wistar rats maintained on this diet can produce anomalous offspring, it seems probable that the strain difference of rats reared on this diet is expressed rather in the growth and attainment of sexual maturity than in the production of anomalous progeny.

The adult female Wistar rats fed on the deficient diet exhibited disturbed reproductive activity as evidenced by small and infrequent litters and by prolonged and irregular vaginal smear cycles. The thyroid glands showed some hyperplasia and increase in weight. Aside from the hypertrophied interstitial tissues, no other qualitative differences were observed in the ovaries. The histologically normal uteri were diminished in weight. The pituitary, thymus, and adrenal glands were similar to those of the controls both in weight and histology. The gonadotrophic activity of the hypophyses of control animals and the experimental animals was comparable.‖

Summary. Gross anatomical malformations can be induced in approximately one-third of the offspring of Wistar rats, if they are fed a deficient diet after maturity. In agreement with previous reports, immature Wistar rats reared on the same diet grow poorly and give little evidence of reaching adult stature and sexual maturity. It has been demonstrated in the literature, however, that immature female rats of either the Sprague-Dawley or Baltimore strains, reared on this diet, attain sexual maturity and produce litters with anomalous young.

It is suggested that the strain differences of rats reared on this diet is expressed rather in the growth and the attainment of sexual maturity than in the production of anomalous offspring.

§ Remington[5] reports similar results but he does not specify the rat strain used.

‖ The fresh hypophyses were triturated and assayed according to the method of Kupperman, H. S., Elder, W., and Meyer, R., *Endocrinology*, 1941, **29**, 23.

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An Enrichment Broth for Isolating Hemolytic Streptococci from Throat Swabs.

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A number of procedures have been described to assist in the isolation of streptococci from mixed cultures. These include methods directed towards the elimination of *Hemophilus hemolyticus*,[1,2] the use of crystal violet to inhibit staphylococci,[3–6] and the use of potassium tellurite[4,7] or sodium azide[6–10] to inhibit Gram-negative bacteria. We have observed that hemolytic streptococci in blood broth tend to outgrow other streptococci and *Neisseria* found in throat cultures. Taking advantage of this fact as well as the selective inhibitory properties of crystal violet and sodium azide, it has been possible to increase the number of hemolytic streptococcus isolations from throat swabs. Without the inhibitors the rapid growth of a few staphylococci or coliform bacilli present on the swabs usually obscured the hemolytic streptococci completely.

Beef heart infusion agar plates containing 5% rabbit blood were inoculated directly with throat swabs. The inoculum was spread with a loop to obtain isolated colonies. The same swabs were then placed in tubes of blood infusion broth prepared as follows. Beef heart infusion broth containing 1% tryptose, 0.02% glucose, and 5% rabbit blood was tubed in 2 ml amounts. On the day the medium was used, to each tube was added 0.15 ml of an autoclaved aqueous solution of sodium azide, 1:1000, and 0.1 ml of an autoclaved aqueous solution of crystal violet, 1:25,000. Experiments showed that the broth began to lose its growth-inhibiting properties if allowed to stand for 2 days after the addition of the sodium azide. Swabs were cultured within one hour after being taken. After incubation overnight, subcultures were made on blood agar plates by streaking from the broth with a straight needle. Hemolytic streptococci were isolated and Lancefield serological groups were determined with formamide extracts.

Results. Staphylococci, *Neisseria*, *Hemophilus*, and coliforms were largely inhibited in the broth cultures. Although alpha and gamma streptococci appeared in large numbers on most of the plates streaked from broth, the isolation of beta hemolytic streptococci was greatly facilitated.

Table I summarizes the results with 131 throat swabs* from well children taken during June and the first week of July. The total number of positive cultures was increased from 16 to 51 by the use of broth; the number positive for Group A from 6 to 26, and for Group C from 8 to 17. The increase in total positive cultures and in those positive for Group A is statistically significant. The number of hemolytic streptococcus colonies on each plate sufficiently separated to be fished directly was noted. The proportion of positive plates with 5 or more such colonies was only 24% on direct plating as compared with 60% for plates streaked from broth. Also, hemolytic streptococci predominated on 23% of the positive plates from broth, while none of the direct plates showed a preponderance

1 Mueller, J. H., and Whitman, L., *J. Bact.*, 1931, **21**, 219.

2 Krumwiede, E., and Kuttner, A. G., *J. Exp. Med.*, 1938, **67**, 429.

3 Edwards, S. J., *J. Comp. Path. Therap.*, 1933, **46**, 211.

4 Rose, K. D., and Georgi, C. E., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 344.

5 Fleming, A., *Brit. Med. J.*, 1942, **1**, 547.

6 Stiles, M. H., and Chapman, G. H., *J. Bact.*, 1942, **43**, 64.

7 Fleming, A., *J. Path. Bact.*, 1932, **35**, 831.

8 Snyder, M. L., and Lichstein, H., *J. Inf. Dis.*, 1940, **67**, 113.

9 Lichstein, H., *J. Bact.*, 1941, **42**, 293.

10 Packer, R. A., *J. Bact.*, 1943, **46**, 343.

* The author is indebted to Dr. Gladys Fashena, Department of Pediatrics, for this material.

TABLE I.
Isolation of Hemolytic Streptococci in 131 Throat Cultures.

	Plates inoculated directly from swabs	Plates streaked from broth cultures
Total positive cultures	16 (12.2%)	51 (38.9%)
Group A	6 (4.6%)	26 (19.8%)
" C	8	17
" F	0	2
" G	3	4
Not grouped	0	2

of hemolytic streptococci. In one instance both Group A and Group C organisms were isolated from the direct plate but only the C was recognized on the plate from broth. From another swab, the direct plate yielded Group A and the plate from broth, Group C.

The Group A carrier rate of 19.8% in this limited series is high as compared with that recorded by other investigators.[11-14] Further studies, now in progress, may indicate an even higher rate in winter months.

Summary. By culturing 131 throat swabs from normal children in a partially selective broth prior to plating, the observed hemolytic streptococcus carrier rate was tripled. Group A streptococci were isolated from 19.8% of the swabs.

[11] Hare, R., *Lancet*, 1940, **1**, 109.

[12] MacDonald, I., Simmons, R. T., and Keogh, E. V., *Med. J. Australia*, 1940, **1**, 849.

[13] Rantz, L. A., *J. Inf. Dis.*, 1941, **69**, 248.

[14] Schwentker, F. F., Janney, J. H., and Gordon, J. E., *Am. J. Hyg.*, 1943, **38**, 27.

14748

Sesame Protein in Chick Diets.

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In a previous note[1] the mutual supplementary action of soybean meal and sesame meal was reported, together with the results of preliminary experiments with sesame protein as the only source of protein in the diet of the chick.

The first experiment of the present series (Table I) was carried out concurrently with the last experiment in the previous report. To the basal diet in which sesame meal was the only protein source, 10% of casein, gelatin, or zein was added. After the chicks had been receiving the diets for 4 days, it appeared that the zein was ineffective in increasing the growth rate over that observed on the basal diet, while casein promoted optimal and gelatin slightly suboptimal growth. From these results and from recent data on the amino acid content of casein,[2] gelatin,[2] and zein,[3] a lysine deficiency in sesame meal was suspected (Table I). On the fourth day of the experiment, *l*(+)-lysine monohydrochloride was added to the zein-supplemented diet to provide 0.9% of the free amino acid. After 24 hours, the chicks in this group had gained enough to make their growth curve parallel to that of the casein group, and they gained at this rate for 4 more days. When the chicks were then returned to the unsupplemented diet, growth practically ceased, as in the beginning of the experiment. When 0.45% lysine was added after 5 more days, the growth

[1] Almquist, H. J., and Grau, C. R., *Poultry Sci.*, 1944, **23**, 341.

[2] Block, R. J., and Bolling, D., *J. Am. Dietetic Assn.*, 1944, **20**, 69.

[3] Vickery, H. B., *Compt. Rend. trav. lab. Carlsberg, Ser. chim.*, 1938, **22**, 519.

TABLE I.
Effect of Protein Supplements on Diets Containing 20% Sesame Meal Protein. There were 8 chicks per group; the experiment lasted 16 days.

Diet	Total gain g	Rate of gain per day %	Lysine added by supplement to diet %
Basal	18	1.5	—
" + 10% casein	148	6.5	0.62
" + 10% gelatin	103	5.3	0.42
" + 10% zein	*	0.3 (days 1-4) 0.8 (" 9-14)	0.03

* This pen was used to study the effects of lysine supplements. See text.

TABLE II.
Effect of Lysine Addition to Diets Containing 20% Raw Sesame Seed Protein. There were 8 chicks per group; the experiment lasted 10 days.

Diet	Total gain g	Rate of gain per day %	Gain per g feed eaten g
Basal	41	3.2	0.112
Basal + 0.5% l (+)-lysine	104	7.4	0.470

curve again became parallel to that of the casein group.

As the partial lysine deficiency of sesame meal became apparent, it seemed possible that the protein might have been heat damaged during oil removal, since the resulting meal was of a dark brown color, and the overheating of proteins is known to reduce the availability of lysine.[4] Intact sesame seeds were ground, extracted with ethyl ether and reground, yielding a product tan in color and containing 40.0% protein (N × 6.25).* When fed to provide 20% protein, raw sesame seed also appeared to be deficient in lysine, since supplementation with pure lysine was necessary for the attainment of optimal chick gains (Table II).

The chick requires approximately 0.9% of the natural form of lysine in the diet.[5] Since half this amount is sufficient to supplement sesame protein for optimal growth, the sesame protein at a 20% level apparently contributes at least half of the lysine requirement. This estimate is also indicated by the results of casein and gelatin supplementation in the first experiment (Table I).

Upon completion of the chick tests, data on the amino acid content of sesame seed proteins became available.‡ These are listed in Table III. The lysine content reported is sufficient to furnish 0.56% lysine to a diet containing 20% sesame protein, which is in good agreement with the estimate based on chick growth results.

The requirements of the chick for arginine,[6]

TABLE III.
Amino Acids in Sesame Seed Proteins.†

Amino acid	Amino acid in the crude protein (N × 6.25) %
Arginine	8.7
Histidine	1.5
Lysine	2.8
Tyrosine	4.3
Tryptophane	1.8
Phenylalanine	8.3
Cystine	1.3
Methionine	3.1
Threonine	3.6
Leucine	7.5
Isoleucine	4.8
Valine	5.1
Glycine	9.3

[4] Greaves, E. O., Morgan, A. F., and Loveen, M. K., *J. Nutr.*, 1938, **16**, 115.

* The sesame seeds were kindly donated by the Pacific Vegetable Oil Corp., through the courtesy of Mr. B. T. Rocca, President.

[5] Almquist, H. J., and Mecchi, E., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 174.

‡ The analyses were reported to us by Dr. R. J. Block. Cost of the analyses was supported by the Pacific Vegetable Oil Corporation and W. R. Grace & Co., San Francisco.

histidine,[6,7] tyrosine and phenylalanine,[7] tryptophane,[6,8] methionine plus cystine,[9] threonine,[7] leucine,[7] isoleucine,[7,10] valine,[7] and glycine,[7,11] are, evidently, approximately met by the quantities of these amino acids in sesame seed protein when it is fed at a level of 20% of the diet. In conjunction with other proteins which are relatively richer in lysine (*i.e.*, soybean meal, fish meal) sesame seed meal is capable of supporting optimal chick growth rates.

[6] Klose, A. A., Stokstad, E. L. R., and Almquist, H. J., *J. Biol. Chem.*, 1938, **123**, 691.

[7] Almquist, H. J., and Grau, C. R., *J. Nutrit.*, in press.

[8] Grau, C. R., and Almquist, H. J., *J. Nutrit.*, in press.

[9] Grau, C. R., and Almquist, H. J., *J. Nutrit.*, 1943, **26**, 631; Klose, A. A., and Almquist, H. J., *J. Biol. Chem.*, 1941, **138**, 467.

[10] Grau, C. R., and Almquist, H. J., *Poultry Sci.*, in press.

[11] Almquist, H. J., and Mecchi, E., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 541.

14749

Effect of Cadmium and Fluorine on the Rat Dentition.

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Wilson *et al.*[1] reported that the addition of cadmium to the diet of rats caused a loss of tooth pigmentation. According to them, the condition resembled the mottled enamel that results from ingestion of excessive amounts of fluorine. Although the latter relationship has been established, the nature of the mechanism by which the pigmentary changes are produced is not clearly understood. Our interest was aroused by the apparently isolated observation of the same authors that cadmium ingestion also produces a marked fall in blood hemoglobin values. Since hemoglobin is an iron-containing protein and since there is evidence that rat incisor enamel pigments are of a similar structure,[2] it seemed plausible that both observations might be related to the ability of cadmium to interfere with normal iron metabolism. Likewise, it seemed possible that the pigmentary changes in fluorosis could be explained on a similar basis. Partial confirmation of this belief would be enhanced by the demonstration of a depression of blood hemoglobin following the ingestion of fluorine.

At the same time it appeared logical to test the ability of cadmium to inhibit experimental rat caries, especially since it has been shown that fluorine can effect such a change. Cadmium in addition to producing mottled enamel also resembles fluorine in its ability to inhibit various enzyme systems. Included among these are those concerned with lactic acid fermentation,[3] a process believed by some to be of considerable importance in the etiology of dental caries.

In addition it seemed opportune to make certain supplementary observations as to the effect of cadmium on the general appearance, body weight, and incisor eruption rate of the rat.

Methods. At weaning 4 groups of rats each containing 18 littermates were placed on a modified Hoppert-Webber-Canniff diet[4] (Comet brand rice was substituted for the coarse corn component).

Group I served as a control. Groups II, III, and IV were maintained on the same diet but received 50 p.p.m. of cadmium ($CdCl_2$) in the food, 50 p.p.m. of cadmium ($CdCl_2$) in

[1] Wilson, R., DeEds, F., and Cox, *J. Pharm. and Exp. Therap.*, 1941, **71**, 222.

[2] Ratner, S., *J. Dental Res.*, 1935, **15**, 89.

[3] Athanasui, J., and Langlois, P., *Arch. Physiol.*, 1896, **8**, 251.

[4] Hoppert, C. A., Webber, P. A., and Canniff, T. L., *Science*, 1931, **74**, 77.

TABLE I.
Hemoglobin of Rats Receiving Cd and F.
(Average grams per 100 cc blood.)

Determination	Group I Control	Group II 50 p.p.m. Cd in diet	Group III 50 p.p.m. Cd in H_2O	Group IV 50 p.p.m. F in H_2O
1st (30th day)	15.8	14.3	11.6	15.2
	(18)*	(18)	(18)	(18)
2nd (44th day)	15.8	13.3	10.8	14.7
	(18)	(18)	(18)	(18)
3rd (65th day)	15.6	12.7	8.7	11.8
	(18)	(17)	(18)	(18)
4th (86th day)	15.8	12.6	7.7	11.1
	(5)	(16)	(7)	(17)

* Numbers in parentheses indicate animals from which average was obtained.

TABLE II.
Effect of Dietary Cd and F on Experimental Rat Caries.

	% of rats with caries	Avg No. of carious teeth	Avg No. of areas affected by caries	Avg total caries score*
Group I Control diet	100	3.5	6.2	12.1
Group II Control diet + 50 p.p.m. Cd in food	100	3.2	6.2	13.0
Group III Control diet + 50 p.p.m. Cd in water	100	5.3	13.8	34.9
Group IV Control diet + 50 p.p.m. F in water	39	.7	1.7	4.4

* For a detailed description of the calculation and significance of the total caries score see Dale, P., and Powell, V. J., *J. Dent. Research,* 1943, **22**, 33.

the water, and 50 p.p.m. of fluorine (NaF) in the water, respectively. The incisor teeth were examined periodically for evidence of mottling. After the animals had been on the diet for 30 days hemoglobin values (Sahli) were determined on blood samples taken from the tail vein. The procedure was repeated on the following 14th, 35th, and 56th day. Also beginning on the 30th day the upper and lower incisor teeth were notched at the gingivolabial margin and the eruption rate measured at suitable intervals over a period of 7 weeks. The weight of the animals was recorded weekly. At the end of 150 days the animals were sacrificed and the dentition examined microscopically for evidences of dental caries.

Results. The observation of Wilson *et al.* that cadmium produced a loss of rat incisor enamel pigment was confirmed. Although the pigmentary changes induced by dietary fluorine were characterized by alternate pigmented and non-pigmented striations, the addition of cadmium to the diet resulted in an overall bleaching devoid of striations. The finding that cadmium produced a significant drop in blood hemoglobin has also been substantiated. A similar change has been observed in rats receiving fluorine. The most marked decrease was evident in Group III animals. The results may be seen in Table I.

The effect of cadmium and fluorine on caries susceptibility is presented in Table II. It can be seen that unlike fluorine, cadmium did not inhibit experimental rat caries. If anything, 50 p.p.m. of cadmium in the drinking water apparently increased caries susceptibility.

Rats in Groups I, II, and IV showed a normal and comparable gain in weight throughout the experimental period. After 8 weeks on the diet, the average weight of these groups ranged between 162 and 172 g. After a comparable period Group III animals weighed on an average of 127 g and the weight differences became more marked with increased age. The incisor eruption rates were comparable in all groups, ranging from 2.1 to 2.2 mm per week for the upper incisors and 3.1 to 3.2 mm for the lower incisors. Group III rats showed lessened physical activity, had dry and scaly skin and tails, and

thinning of the hair especially in the posterior portion of the dorsum.

Discussion. It is of interest that both cadmium and fluorine caused a loss of incisor enamel pigmentation and a drop in blood hemoglobin indicating in each instance an effect on normal iron metabolism. Although the exact mechanism of the interference is in need of further clarification, the ability of cadmium to precipitate iron-containing proteins has been noted[5] and it has been observed that the iron content of fluorosed enamel is markedly diminished.[6]

The failure of dietary cadmium to limit experimental caries in the same manner as fluorine suggests that the caries limiting property of the latter is not related to the inhibition of lactic acid fermentation by oral organisms. Likewise, it does not appear to be dependent on an inhibitory action on bone phosphatase since it has been shown that both cadmium[1] and fluorine[7] limit the activity of this enzyme.

Summary. The ability of cadmium to diminish the degree of pigmentation of rat incisor enamel and to effect a reduction in blood hemoglobin has been confirmed. The ability of fluorine to bring about a comparable reduction in blood hemoglobin has also been observed. It is suggested that the pigmentary changes in the enamel and the fall in hemoglobin that results in the ingestion of both cadmium and fluorine is related to the ability of these elements to interact with iron-containing proteins. Unlike fluorine, cadmium in the concentration studied is ineffective in reducing experimental rat caries.

5 Granick, S., and Michaelis, L., *Science*, 1942, **95**, 439.

6 Bowes, J. ., and Murray, M. M., *Brit. Dent. J.*, 1936, **60**, 556.

7 DeEds, F., *J. A. D. A.*, 1936, **23**, 568.

14750

Glycogen in Alloxan-Treated Rats.

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Fisher and Lackey[1] found the glycogen content of the heart of the dog to be increased following pancreatectomy. Evans *et al.* found a similar increase in cardiac glycogen in 3 depancreatized cats.[2] Since, in these experiments, both exocrine and endocrine secretions of the pancreas were eliminated, it seemed desirable to study the glycogen changes in the heart when the endocrine secretion alone is disturbed. The demonstration by Dunn and McLetchie[3] and Gomori and Goldner[4] that diabetes mellitus can be produced in rats by a single injection of alloxan, and that this chemical destroys specifically the islet tissue of the pancreas, makes it possible to carry out such a study. Also, alloxan makes it possible to study diabetes mellitus in the rat, an animal in which surgical removal of the pancreas is very difficult.

Method and Results. The glycogen content of liver, heart, and skeletal muscle was determined in 20 control animals (Table I) and 19 animals with alloxan diabetes (Table II). The animals used were full grown rats of approximately the same weight. Three rats in each series were females and the remainder were males. Each animal was kept in a separate metabolism cage with ample food and water.

1 Fisher, N. F., and Lackey, R. W., *Am. J. Physiol.*, 1925, **72**, 43.

2 Evans, Gerald, and Bowie, Morris A., PROC. SOC. EXP. BIOL. AND MED., 1936-37, **35**, 68.

3 Dunn, J. Shaw and McLetchie, G. B., *Lancet*, 1943, **245**, 384.

4 Gomori, G., and Goldner, M. G., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 287.

Alloxan was injected as a freshly prepared 5% solution. 200 mg of alloxan monohydrate per kilogram of body weight was given as a single dose intraperitoneally. Not all animals injected with alloxan developed diabetes as determined by 2 daily urine tests for ketones and sugar. Those animals which showed neither glycosuria nor ketonuria were not used for glycogen analyses. Some alloxan-injected rats died or were in a moribund state before tissues were obtained for analysis. These, too, were discarded. Therefore, the 19 animals in Table II represent all alloxan-injected animals which survived three or more days and developed moderate to severe diabetes, without becoming moribund.

At autopsy rats were injected with sodium pentobarbital, which has been shown to have no effect on the glycogen content of tissues.[5] While anesthetized, a lobe of the liver, the entire heart, and a portion of thigh muscle were quickly removed and cut into small pieces with scissors and immersed in hot potassium hydroxide. The entire procedure for all 3 tissues was completed in less than one minute. Blood was also obtained for ketone and glucose determinations in animals C6 to C21 and A19 to A33. Glycogen analyses were done according to the method of Good, Kramer, and Somogyi.[6] The determination of glucose obtained from hydrolysis of the glycogen was made by the method of Shaffer and Somogyi.[7] The blood ketones were determined by the method described by Weichelbaum and Somogyi,[8] and the blood sugars by the method of Hagedorn and Jensen.[9]

After removal of tissues for glycogen analysis, the pancreas, one kidney, and portions of the liver and heart were removed and prepared for histological study. Tissues were fixed immediately in 10% formalin. After fixation, the tissues were imbedded in paraffin and sectioned at 7 microns. All sections were stained with Harris' hematoxylin and eosin.

In Tables I and II are summarized the

TABLE I.
Glycogen Content of Liver, Heart, and Skeletal Muscle in Untreated Adult Rats.

Rat	% Liver glycogen	% Heart glycogen	% Muscle glycogen
CE 5	4.2	.34	.52
1	3.48	.29	.57
C 2	3.42	.43	.57
5	1.88	.40	.396
6	2.88	.31	.568
7	4.41	.68	1.02
8	3.7	.43	.39
3	1.63	.24	.47
9	4.15	.47	.54
10	3.71	.44	.54
11	3.83	.56	.59
12	3.18	.42	.51
13	3.10	.41	.45
14	1.24	.53	.476
15	1.58	.60	.42
16	4.24	.518	.58
17	6.39	.49	.51
18	1.164	.462	.405
19	1.13	.408	.354
21	1.819	.410	.434
Mean	3.056 ± .317*	.442 ± .024*	.515 ± .032*

* Standard error.

TABLE II.
Glycogen Content of Liver, Heart, and Skeletal Muscle in Adult Rats Having Alloxan-Produced Diabetes Mellitus.

Rat	% Liver glycogen	% Heart glycogen	% Muscle glycogen
A 1	.091	.582	.138
3	1.01	.827	.309
8	.79	1.24	.36
9	.68	.88	.43
10	.25	.73	.37
12	.05	.63	.06
13	.14	.82	.092
17	.248	1.0	.028
19	1.39	.95	.56
20	1.37	.32	.49
21	1.28	1.19	.31
22	1.23	1.21	.37
23	.72	.77	.32
25	.95	1.22	.59
27	.98	.87	.28
29	.65	.735	.066
30	1.76	.604	.543
32	.0795	.814	.391
33	.833	.834	.473
Mean	.833 ± .122*	.854 ± .056*	.325 ± .040*

* Standard error.

5 Shelley, W. B., Code, C. F., and Visscher, M. B., *Am. J. Physiol.*, 1943, **138**, 652.

6 Good, C. A., Kramer, H., and Somogyi, M., *J. Biol. Chem.*, 1933, **100**, 485.

7 Shaffer, P. A., and Somogyi, M., *J. Biol. Chem.*, 1933, **100**, 695.

8 Weichelbaum, T. E., and Somogyi, M., *J. Biol. Chem.*, 1941, **140**, 5.

9 Hawk and Bergeim, *Practical Physiological Chemistry*, P. Blakiston's Son and Co., Inc., Philadelphia, Pa., 1937, p. 439.

analytical data on tissues of normal rats and alloxan-treated rats respectively. A comparison of these tables shows that alloxan-injected rats have an elevated heart glycogen and a decreased glycogen content of both liver and skeletal muscle. The mean value for heart glycogen in control animals was .442%, and for alloxan-treated animals was .854%. The mean value for liver glycogen of control animals was 3.056%, and for alloxan-treated animals was .806%. The mean value for muscle glycogen of control animals was .515%, and for alloxan-treated animals was .325%. These data were analyzed for significance by the T method of Fischer, and for each tissue a P value of less than .01 was obtained.

The alloxan-injected animals on which blood sugar and ketone determinations were made showed a marked elevation of both. The ketones averaged 29.5 mg % as compared to 3.2 mg % for the controls. Blood sugars averaged 443 mg % in the alloxan group and 136 mg % in the controls.

Discussion. Our experience with alloxan confirmed that of others[3,4,10] in that the animals showed considerable individual variation in susceptibility. Approximately 60% of the animals developed moderate to severe diabetes mellitus. The remaining 40% either died in the first 72 hours or failed to develop any apparent diabetes. In the limited number of females used, no sex difference was observed either in susceptibility to alloxan or in glycogen distribution. Contrary to Gomori and Goldner,[4] we observed no difference in reaction to alloxan between albino and hooded strains of rats. Approximately one-half of the rats used by us were albino and one-half hooded. A most striking polyuria was observed in some of the diabetic animals. In some instances rats weighing 175 to 200 g excreted as much as 60 ml of urine in 24 hours.

The fact that the glycogen content of the heart increases in diabetes, while that of skeletal muscle decreases, suggests some fundamental difference in the metabolism of these two types of muscle tissue. Other conditions such as reduced barometric pressure,[11] and fasting,[12] are reported to alter the glycogen content of these 2 tissues differentially, but no satisfactory explanation has been offered. The most striking metabolic changes between normal and diabetic animals are the great increases in blood sugar and blood ketone levels. Whether or not these changes bear any real relation to the increased heart glycogen in diabetes mellitus, we cannot say on the basis of the present work.

A study of microscopic sections from the alloxan-injected animals sacrificed between the third and seventh days after injection revealed fairly uniform histological changes. In the pancreas under low magnification islets appear fewer in number, more cellular, and smaller than in those of control animals. Higher magnification revealed marked reduction in the amount of cytoplasm of the cells which make up the bulk of each islet. The majority of the cells present are transformed from the usual large beta cells with abundant pale-staining cytoplasm into cells with shrunken, granular, somewhat eosinophilic cytoplasm or with extensive vacuolation of the cytoplasm. Loss or reduction of the amount of cytoplasm is the most outstanding change and causes what we believe is a real collapse of islets so that they appear smaller and more cellular than normal. In some cases this collapse of islets may simulate cellular proliferation. The nuclei tend to be smaller and more pycnotic than those of the original cells, but it is often difficult or impossible to be certain of a real nuclear change even in the cells with markedly depleted or vacuolated cytoplasm.

At this stage (*i.e.*, between third and seventh days) very few of the islet cells in our animals are actually necrotic, although necrosis has been described as occurring within 7 hours of poisoning.[13] Our study in this series does not include the very early stages, and we are not prepared to comment at this time on the origin or the nature of the cellular proliferation which must occur to provide the cells which have replaced the normal parenchyma of islets in our animals studied from 3 to 7

[10] Bailey, C. C., Bailey, O. T., and Leech, R. S., *New England J. Med.*, 1944, **230**, 533.

[11] Kreienberg, W., and Wisehutter, E., *Pfluger's Arch. f. d. Ges. Physiol.*, 1943, **247**, 11.

[12] Evans, G., *J. Physiol.*, 1934, **82**, 468.

[13] Ridout, J. H., Ham, A. W., and Wrenshall, G. A., *Science*, 1944, **100**, 57.

days after alloxan injection. From examination of the tissues in this series it might be suspected that the majority of the cells present are degenerate forms of the original beta cells. There is little difference in the appearance of islets between the third and seventh days. In several alloxan-treated animals (30A, 6A, 26A), the pancreatic islets retain a normal appearance. In each instance where this occurred the animals failed to develop diabetes.

Almost all the sections of kidney in this series of animals with alloxan diabetes showed varying grades of parenchymatous degeneration most notable in epithelium of the convoluted tubules. In several cases there was marked swelling and hydropic degeneration of individual and small groups of epithelial cells. Two animals (29A, 10A) showed marked degeneration of epithelium of both limbs of loops of Henle. In these cases the changes appeared most marked in the ascending limbs where much of the epithelium was necrotic and the lumina were often filled with blue-stained granular debris.

Liver sections revealed frequent widespread parenchymatous degeneration and early stages of hydropic degeneration. No consistent changes were observed in the myocardium.

Summary. Glycogen determinations were made on the heart, liver, and skeletal muscle of rats with alloxan-produced diabetes mellitus and on the same tissues of untreated controls. The alloxan-treated animals showed a statistically significant increase in glycogen content of heart muscle and a statistically significant decrease in liver and skeletal muscle glycogen.

Rats which developed diabetes mellitus had a high blood sugar and high blood ketones at the time of autopsy.

On histological examination of the tissues, the islet cells of alloxan-treated rats which developed diabetes showed a marked reduction in cytoplasm, pycnosis and decrease in size of the nuclei with resultant collapse of islet structure. Almost all kidney and liver sections showed some degenerative changes.

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Effect of Trypan Blue upon Cardiac Explants in Tissue Culture.

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During some previous work with trypan blue in tissue cultures it was observed that the growth of explants in the dye-containing medium appeared to be more luxurious than in the control cultures. When buffy coat[1] of chicken blood was cultivated in a trypan blue medium, monocytes markedly increased in numbers as they became transformed into macrophages. Attempts were made to estimate this increase in numbers of phagocytic cells by making surface counts of macrophages in so many contiguous microscopic fields and comparing them with similar counts made upon control cultures stained by neutral red at the time of counting. There was a noticeable difference, but since cultures of buffy coat did not make a compact or membranous growth on the under surface of the cover-slip, comparative areal measurements could not be made and the matter was dropped.

The following experiment was designed to test the presence or absence of a stimulating effect upon growth of tissue cultures in a medium containing trypan blue.

At any one planting, control and experimental cultures were made on cover-slips from fragments of the same heart removed from a 10-day chick embryo. Lyophiled embryo juice (20%) and plasma diluted to volume with

* The author wishes to express his thanks to Jane Stanley Craig, who assisted in these experiments.

1 Hetherington, Duncan C., *Arch. f. exp. Zellforsch.*, 1931, **11**, 520.

TABLE I.
Showing Statistical Results of Cardiac Explants Grown with Trypan Blue Versus Growth of Controls.

	Control series				Significant difference				Trypan series			
Days of growth	Mean total area in mm²	Probable error of mean	σ	Total growth in %	D_M	σ_D	$3\sigma_D$	Sig. D	Mean total area in mm²	Probable error of mean	σ	Total growth in %
0	0.71	±.01	0.20	0	0.02	0.10	0.30	—	0.73	±.06	0.77	0
1	1.75	±.04	0.53	145	0.19	0.10	0.30	—	1.94	±.05	0.70	165
2	3.26	±.06	0.83	359	0.43	0.19	0.57	—	3.69	±.13	1.39	405
3	5.25	±.13	1.83	639	1.12	0.37	1.11	+	6.37	±.20	2.53	772
4	8.01	±.20	3.28	1028	3.28	0.64	1.92	+	11.29	±.33	4.25	1460
5	11.39	±.34	4.31	1504	7.24	0.86	2.58	+	18.63	±.40	5.76	2452
6	14.22	±.40	5.07	1902	8.49	0.95	2.85	+	22.71	±.47	6.16	3010
7	17.22	±.47	5.72	2325	10.30	1.01	3.03	+	27.52	±.47	6.23	3669
8	21.15	±.53	6.22	2878	10.62	1.10	3.30	+	31.77	±.54	6.78	4252

D_M, difference of the means; σ_D, standard error of the difference of the means; Sig. D, significant difference $= D_M > 3\sigma_D$.

distilled water, were mixed in equal proportions and constituted the medium for growth of all control cultures. The experimental cultures were planted in the same manner except that to the embryo juice trypan blue was added beforehand in the ratio of 1/2000, the final dilution in the cultures becoming 1/4000. Sterile precautions were used throughout and all cultures were incubated at 37.5°C.

Delineascope and planimeter records were made of the original explants and thereafter at 24-hour intervals for 8 days. The results involving the records of 70 cultures in each series were treated statistically and appear in Table I.

The cultures were composed of a mixed population of cells, cardiac muscle, endothelium, mesothelium, a moderate number of macrophages, and predominating fibroblasts. As is common with such mixed groups of cells, the fibroblasts constituted the bulk of the radial outgrowth almost immediately. As far as could be determined microscopically the cell types in the control and trypan blue cultures were comparable except for the differential reaction of the cells in acquiring trypan blue inclusions and a more noticeable number of macrophages in the experimental series. These phagocytic cells did not migrate fast enough to keep up with the outgrowth of fibroblasts so that at no time were they a confusing factor in making the delineascope tracings and therefore did not complicate the marked areal increase noted in the dye cultures.

Only occasionally and usually late in the life of the culture, did cardiac muscle acquire a very few dye inclusions. These could be seen to be moved about in the cytoplasm of the contracting fibers. Mesothelial and endothelial cells which were never very numerous, accumulated only after a time a small number of very fine dye inclusions, arranged usually close to the nucleus. Fibroblasts also took up trypan blue and segregated it into vacuoles of various sizes, but without any patterned orientation within the cytoplasm. Many of these cells contained no dye. The most obviously loaded cells were the macrophages some of which underwent epithelioid transformations toward the end of the culture period. Record keeping was terminated on the eighth day of growth because the dye cultures became patchy rather suddenly and began to die off after this period. This behavior was attributed to the exhaustion of the small amount of food available in an undisturbed hanging drop culture.

It is apparent from Table I that the growth of cardiac explants in trypan blue medium was significantly more copious than in the control cultures. The explanation for this trend is not clear. Some tissues, notably liver, kidney, and spleen, excised from animals which had previously been treated with trypan blue have

[2] Kiyono, K., Sugiyama, S., and Amano, S., *Die Lehre von der Vitalfärbung*, Kyoto, II Hauptteil, 1937, p. 282.

been reported by Kiyono and associates[2] to exhibit an increased oxygen uptake as measured in the Warburg respirometer.

It is possible that the presence of the dye in some way stimulates the oxygen uptake and thus induces an increased growth rate of the cells. This hypothesis is only a tentative one since increased metabolic rate is not necessarily a sign of growth.

Summary. Cardiac explants from 10-day chick embryos planted in a medium of lyophiled chicken plasma and embryo juice containing 1/4000 trypan blue, grew significantly better than control cultures from the same hearts grown in an identical medium without the dye.

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Frozen-Dried Serum as a Medium Constituent for Tissue Cultures.

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It has been demonstrated that frozen-dried chicken plasma and also embryo juice sealed *in vacuo* and stored for variable lengths of time, will, when redissolved to volume with sterile distilled water, form adequate media for growth of tissue cultures from chick embryos.[1] Furthermore a very potent embryo juice was obtained from previously ground, frozen-dried chick embryos. Juice prepared from this dried material by extraction with Ringer-Tyrode's solution can be used at once or can be frozen dried and stored for future use.[2]

Plasma and embryo juice constitute an adequate medium, among others, for cultivation of chick embryo tissues by the cover-glass method where experiments demand maintenance of the cultures for only 7-14 days. Cultivation of mammalian tissue requires a fuller medium met best by the addition to the plasma and embryo juice of serum,[3,4] either homologous or heterologous.

Since lyophiled embryo juice and plasma proved so satisfactory for cultivating chick tissues it was decided to determine what effect frozen-dried serum as an added constituent of the medium, might have upon the growth of cardiac explants from young mice.

Human cord serum was collected on occasions by the method outlined by the Geys.[4] At other times by cooperation of the Department of Obstetrics and Gynecology, when routine cord blood Wassermann samples were taken at the time of delivery, an additional tube was set aside in an ice-box for our use.

The serum, after retraction of the clots, was removed aseptically and pooled. Highly colored, cloudy or hemoglobin tinted serum was discarded. When a sufficient quantity was collected and tested for sterility by bouillon cultures, it was delivered into sterile 1 cc ampoules, lyophiled, and sealed *in vacuo*.

In the following experiments during any one planting of cultures upon cover-slips 3 series were set up. The heart of a 4-weeks-old white mouse was removed aseptically under ether anesthesia and placed in Ringer-Tyrode where it was carefully cut into uniformly small pieces by cataract knives. Cultures of Control A series were planted in a mixture of fresh embryo juice (20% strength prepared from 11-day chick embryos), fresh chicken plasma, and fresh human cord serum. Control B series cultures were set up in fresh serum, and frozen-dried embryo juice (20% made from previously ground frozen-dried 11-day chick embryos) and frozen-dried chicken plasma. In the Experimental series of cultures

* The author wishes to express his thanks to Jane Stanley Craig, who assisted in this work.

1 Hetherington, Duncan C., and Craig, Jane Stanley, PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 831.

2 Hetherington, Duncan C., and Craig, Jane Stanley, PROC. SOC. EXP. BIOL. AND MED. 1940, **44**, 282.

3 Lewis, W. H., *Contrib. to Embryology*, No. 150, Carnegie Inst. of Washington, 1935, **25**, 163.

4 Gey, George O., and Gey, Margaret K., *Am. J. Cancer*, 1936, **27**, 45.

TABLE I.
Statistical Results of Cultures Grown in All Lyophiled Media as Compared with Growth of Control Cultures.

Days of growth	Control A Series: Mean total area in mm²	Probable error of mean	σ	Total growth in %	Control B Series: Mean total area in mm²	Probable error of mean	σ	Total growth in %	Experimental Series: Mean total area in mm²	Probable error of mean	σ	Total growth in %
0	0.85	±.01	0.23	0	0.87	±.02	0.31	0	0.85	±.02	0.26	0
1	1.15	±.02	0.30	35	1.21	±.03	0.37	39	1.23	±.03	0.35	44
2	1.50	±.03	0.37	76	1.69	±.04	0.49	94	1.80	±.04	0.53	112
3	1.97	±.04	0.56	132	2.38	±.05	0.74	173	2.56	±.07	0.86	201
4	2.67	±.05	0.58	214	3.11	±.06	0.95	257	3.64	±.10	1.31	328
5	3.75	±.08	1.04	341	4.38	±.10	1.35	403	5.72	±.11	1.37	573
6	4.99	±.11	1.45	487	5.86	±.11	1.43	573	7.91	±.19	2.39	831
7	6.35	±.16	1.91	647	7.44	±.12	1.53	755	10.07	±.20	2.62	1085
8	8.32	±.16	1.95	879	9.39	±.15	1.92	979	12.14	±.18	2.25	1328

Significant Difference

Days of growth	Control A vs. Control B: D_M	σ_D	$3\sigma_D$	Sig. D	Control A vs. Experiment: D_M	σ_D	$3\sigma_D$	Sig. D	Control B vs. Experiment: D_M	σ_D	$3\sigma_D$	Sig. D
0	0.02	.04	.12	—	.00	.04	.12	—	.02	.04	.12	—
1	0.06	.05	.15	—	.08	.06	.18	—	.02	.06	.18	—
2	0.19	.07	.21	—	.30	.08	.24	+	.11	.08	.24	—
3	0.41	.11	.33	+	.59	.12	.36	+	.18	.13	.39	—
4	0.44	.13	.39	+	.97	.17	.51	+	.53	.19	.57	—
5	0.63	.20	.60	+	1.97	.20	.60	+	1.34	.23	.69	+
6	0.87	.25	.75	+	2.92	.33	.99	+	2.05	.34	1.02	+
7	1.09	.32	.96	+	3.72	.39	1.17	+	2.63	.39	1.17	+
8	1.07	.35	1.05	+	3.82	.35	1.05	+	2.75	.38	1.14	+

D_M, difference of the means; σ_D, standard error of the difference of the means; Sig. D, significant difference $= D_M > 3\sigma_D$.

all of the constituents of the medium were frozen-dried. The lyophiled serum was 2 months old, while the embryo juice and plasma had been kept *in vacuo* for 18 months. In all cultures equal portions of serum, plasma, and embryo juice were used. Cultures were incubated at 37.5°C.

Records were kept by means of delineascope and planimeter of the areas of the original explants; thereafter for 8 days, the total area of each culture was measured at 24-hour intervals. The statistical results of 69 cultures in each series appear in Table I. From these it will be seen that in both the Control B and Experimental series growth was significantly better than the growth in Control A series where all components of the medium were fresh. Significant difference between the Control B and Experimental series appeared only after the first 4 days of growth. It would seem therefore that growth is enhanced by using frozen-dried serum in addition to the lyophiled plasma and embryo juice. The explanation is not clear since freezing-drying is not believed to denature biological material such as antigens, etc.[5] If this be true, fresh plasma and serum may contain some substance or substances acting as mild growth depressants, which during lyophiling are removed by volatilization.

The practical advantage of lyophiled media in tissue culture lies in the fact that with an adequate supply of material on hand much routine labor can be avoided. Furthermore for any given series of comparative experiments a constant medium can be maintained.

Summary. Lyophiled serum, embryo juice and plasma when used together form excellent media for the growth of cardiac explants from white mice. They have the added advantage of forming a standardized medium for comparative experiments.

[5] Elser, William J., Thomas, Ruth A., and Steffin, Gustav I., *J. Immunol.*, 1935, **28**, 433.

14753

Pituitary Stimulating Property of Stilbestrol as Compared with That of Estrone.

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The synthetic compound, 4,4′-dihydroxy-α,β-diethylstilbene (stilbestrol), appears to have all of the biological properties of the naturally-occurring estrogens. It is a potent estrogen, considerably less of it than of estrone being required to produce estrus in spayed female rats. It is known to cause the stimulation and release of pituitary adrenotropic and gonadotropic hormones when administered to rats over short intervals of time, as reflected by ovarian, adrenal, and pituitary hypertrophy and a decrease in the transplantable gonadotropic activity of the hypophysis.[1,2] No work has been reported, however, revealing whether more or less of it than of the naturally-occurring estrogens is required for these pituitary stimulating effects.

In a recent publication[3] results were presented indicating that estrone elicits these pituitary responses in mature male rats indirectly through the formation of inactivation products. It was shown that the presence of the testes or the administration of progesterone, both of which depress estrogen inactivation, completely inhibited the hypophyseal reactions to an otherwise effective dose of estrone. It was also demonstrated that Westerfeld's lactone,[4] an oxidative inactivation product of estrone, exerted these same influences upon the pituitary gland and in much smaller dosages than estrone, and, furthermore, that the responses to the lactone were uninfluenced by the presence of the testes or the administration of progesterone. Inasmuch as a compound chemically similar to Westerfeld's lactone could not possibly be formed in the metabolic inactivation of stilbestrol, it was considered of interest to investigate further the pituitary stimulating property of this estrogen.

Exactly the same procedure as previously described[3] was followed, the stilbestrol being given in 10 injections over 5 days to mature (70- to 100-day-old) male rats whose adrenals and pituitaries were weighed on the sixth day and the pituitaries transplanted into immature female rats in order to measure their gonadotropic activity. For each test donor an uninjected littermate was simultaneously autopsied and the results expressed in terms of percentage difference between the weights of the adrenals and pituitary of the injected animal and those of its control littermate. The pituitaries of 2 test donors were injected into an immature female rat, a control littermate of which received 2 pituitaries from uninjected donors. The gonadotropic activity was gauged by the percentage difference between the weight of the ovaries of the test animal and that of its control littermate. From the results of the previously published control experiments,[3] no increase of less than approximately 10% in the adrenal or pituitary weights or decrease of less than 10% in ovarian weights is significant.

In the earlier investigation[3] it was found that whereas a total dose of 10 γ of estrone to *castrated* rats caused a significant adrenal enlargement, as compared with castrated controls, and decrease in the transplantable gonadotropic activity of the pituitary, 120 γ had no demonstrable effect upon the pituitaries or adrenals of *intact* animals. Fifty γ of estrone alone to castrates caused a significant increase in pituitary weights as well as a significant enlargement of the adrenals and decrease in transplantable gonadotropic activity of the pituitaries, but if a total dose of 1.0 mg of progesterone was simultaneously ad-

[1] Von Haam, E., Rardin, T. E., and Schoene, R. H., *Endocrinology*, 1941, **28**, 263.

[2] Wolfe, J. M., and Brown, A. D., *Endocrinology*, 1942, **31**, 467.

[3] Smith, O. W., *Endocrinology*, 1944, **35**, 146.

[4] Westerfeld, W. W., *J. Biol. Chem.*, 1942, **143**, 177.

TABLE I.
Adrenal and Pituitary Weights and Transplantable Gonadotropic Activity of the Hypophyses of Mature Male Rats After Estrone.

Total dose of estrone, γ	No. of exp.	% change in organ weights vs. controls: Donor's adrenals (avg)	Donor's pituitaries (avg)	Recipient's ovaries (avg)
A. Estrone alone to intact animals.				
10	4	+ 5.7	+ 0.7	+ 1.5
50	6	+ 3.2	+ 2.4	— 3.7
100-120	4	— 1.1	— 3.2	+ 0.5
500	4	+30.3	+17.7	—24.2
B. Estrone alone to castrated animals.				
5	4	+ 5.2	— 1.5	—10.7
10	4	+20.7	+10.3	—47.4
50	4	+29.9	+33.8	—39.5
100	2	+40.2	+27.1	—45.5
C. Estrone plus progesterone (1.0 mg) to castrated animals.				
50	6	+ 5.7	— 0.9	— 2.9

TABLE II.
Adrenal and Pituitary Weights and Transplantable Gonadotropic Activity of the Hypophyses of Mature Male Rats After Stilbestrol.

Total dose of stilbestrol, γ	No. of exp.	% change in organ weights vs. controls: Donor's adrenals (avg)	Donor's pituitaries (avg)	Recipient's ovaries (avg)
A. Stilbestrol alone to intact animals.				
1	4	+ 5.3	+ 8.4	+ 3.3
5	4	+33.7	+12.7	—35.6
10	4	+40.1	+17.9	—31.5
B. Stilbestrol alone to castrated animals.				
1	4	— 1.5	+ 6.3	+ 3.1
5	4	+32.1	+31.0	—29.5
10	4	+27.1	+26.5	—21.5
25	2	+31.3	+26.9	—34.5
50	2	+32.0	+24.7	—29.0
C. Stilbestrol plus progesterone (1.0 mg) to castrated animals.				
10	4	+22.0	+32.6	—25.0
50	4	+43.2	+25.3	—28.4

ministered, the pituitary responses of castrated male rats to 50 γ of estrone were completely nullified. In Table I these reported results are summarized and 10 additional experiments are included demonstrating that 500 γ of estrone are required to elicit significant changes in intact rats and that 10 γ is the minimal effective dose in castrates.

The results of 36 experiments with stilbestrol are summarized in Table II. The minimal effective dose of this synthetic estrogen to both castrated and intact rats was 5 γ, the only difference being that the pituitary enlargement was less marked in intact animals. The simultaneous administration of a total dose of 1.0 mg of progesterone had no demonstrable effect upon the pituitary responses of castrated males to 10 or 50 γ of stilbestrol.

Stilbestrol, therefore, appears to be about 100 times as active as estrone in eliciting the pituitary responses of intact rats, although only about twice as active in castrated animals. The fact that its effectiveness is uninfluenced by the presence of the testes or the administration of progesterone indicates, as would be expected, that its metabolism is not regulated by the same mechanism as the naturally-occurring estrogens. Possibly its effect upon the pituitary gland is direct rather than through the formation of some metabolic inactivation product, as appears to be the case with estrone. The results reported shed no

light upon the mechanism of its action but suggest that, as a therapeutic agent in women with hypo-pituitary-ovarian disorders, it should be very much more effective than the naturally-occurring estrogens. The clinical experience of one of us (G.V.S.) corroborates this.

Summary. Stilbestrol is about 100 times as active as estrone in causing the stimulation and release of adrenotropic and gonadotropic factors from the pituitaries of intact mature male rats. In castrated animals it is only about twice as active as estrone. Unlike estrone, the pituitary responses to stilbestrol are not influenced by the presence of the testes or the administration of progesterone, both of which depress the rate of inactivation of estrone as well as its pituitary stimulating activity. It would appear that the mechanism of stilbestrol's action upon the pituitary differs from that of the naturally-occurring estrogens.

14754

Antigenic Types of *Shigella alkalescens.*

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Until 1939, *Shigella alkalescens,* a member of the *Shigella* group, was considered to be an antigenically homogeneous species. In that year Assis[1] described as *S. alkalescens* type II a strain (No. 648) which differed both in biochemical characteristics and antigenic structure from the hitherto known strains comprising type I. In 1943, Stuart, Rustigian, Zimmerman, and Corrigan[2] demonstrated that type I strains contain 5 antigenic components, which they designated A, B, C, D and E. The major antigens A and B and the minor antigen C are present in all typical strains. The antigenic fractions D and E were found either singly or in combination. In May, 1944, Captain William H. Ewing, Sanitary Corps, United States Army, sent several strains, identified as *Shigella,* to the author to determine whether some of them may be identical with type II of *S. alkalescens.* Serological studies carried out in this laboratory revealed that Ewing's strain No. 2-193 differed from both types I and II of *S. alkalescens.* While these studies were in progress a *S. alkalescens* strain (No. 9731) was isolated from the feces of a healthy infant, which was not agglutinated to titer by antisera to types I and II as well as strain No. 2-193. The experiments reported in this communication reveal that strains No. 9731 and No. 2-193 represent hitherto undescribed types of *S. alkalescens.*

Material and Methods. The following strains were employed: (1) Strain No. 1-2, *S. alkalescens* type I, received from Captain Ewing; (2) Strain No. 648, *S. alkalescens* type II, obtained through the courtesy of Dr. Arlindo de Assis; (3) No. 2-193 sent by Captain Ewing; (4) No. 9731 isolated at this laboratory from the feces of a healthy infant.

Antisera were prepared in rabbits. Two rabbits were injected intravenously with 3 ml of each bacterial suspension. Two injections, one week apart, were given. Ten days after the last injection the animals were bled and the serum was collected.

In addition to these sera, 2 anti-*S. alkalescens* type I sera were employed: (1) No. 842 prepared at this laboratory and (2) a serum obtained through the courtesy of Dr. E. G. E. Murray, McGill University, Montreal.

Sera used in the slide agglutination test for the determination of the various types belonging to the genus *Shigella* were procured

[1] Assis, A., *O Hospital,* 1939, **15**, 447 and 655.

[2] Stuart, C. A., Rustigian, R., Zimmerman, A., and Corrigan, F. V., *J. Immunol.,* 1943, **47**, 425.

TABLE I.
Agglutinin Titer of Different *S. alkalescens* Sera.

S. alkalescens sera	Strains of *S. alkalescens*			
	Type 1 No. 1-2	No. 2-193	No. 9731	Assis type II
Type I (No. 1-2)	*1:1600*	1:10	1:100	1:10
" I (No. 842)	*1:1000*	<1:10	<1:10	<1:10
" I (McGill)	1:1000	<1:10	1:100	<1:10
No. 2-193 (a)	1:10	*1:2000*	1:10	1:80
(b)	<1:10	*1:4000*	1:20	1:160
No. 9731 (a)	<1:10	<1:10	*1:1000*	<1:10
(b)	1:10	<1:10	*1:8000*	1:20
Assis type II	1:80	1:320	1:320	*1:4000*

through the kindness of Dr. A. J. Weil, Lederle Laboratories, Pearl River, New York.

Unless otherwise indicated, all agglutination and agglutinin-absorption tests were carried out in test tubes 100 mm in length and 13 mm in width. Serial dilutions of the respective sera (1:10, 1:20, etc.; 1:100, 1:200, etc.; 1:1000, 1:2000, etc.) were prepared, using different pipettes for every dilution. As antigens, bacteria suspended in 0.4% formaldehyde-saline solution were used. The resulting agglutination was read with the naked eye after various periods of incubation at 55°C.

Results. The 4 strains under investigation (No. 1-2, No. 2-193, No. 9731, and Assis type II) were studied with respect to morphology, motility, and biochemical characteristics. The organisms are gram-negative bacilli. They grow profusely in infusion broth as well as on Endo agar. They are non-motile. The colonies on plain agar are smooth. With the exception of Assis type II these strains were found to be identical in biochemical activities. They produced acid without gas from glucose, maltose, mannitol, rhamnos, xylose, dulcitol, and sorbitol. They failed to form acid from lactose, sucrose, and salicin. Gelatin was not liquefied. Indole was formed. In litmus milk they first produced acid and later alkaline reaction. Strain No. 648, type II of Assis, differed from the other strains in its inability to produce acid from rhamnose and dulcitol and its capacity to form acid from salicin. In view of these differences in biochemical activity the question arises as to whether or not this strain will ultimately be classified as *S. alkalescens;* rather, it may belong to *S. paradysenteriæ.* Thus, biochemically, the hitherto undescribed strains No. 2-193 and No. 9731 present the pattern of *S. alkalescens* type I.

The antigenic structure of these strains was investigated by means of agglutination tests. The agglutinin titer of sera against the 4 strains was determined. The experiments were carried out in triplicate. Final readings were made after incubation at 55°C for 18 hours. The results are presented in Table I.

It may be seen from this table that all sera tested agglutinated the homologous strains in titer ranging from 1:1000 to 1:8000. It is also evident that strains No. 2-193, No. 9731 and Assis type II were agglutinated only to a fraction of the titers of three Type I antisera. This indicates that strains No. 2-193 and No. 9731 differ in their major antigens from *S. alkalescens* types I and II. Furthermore, it is clear that the hitherto undescribed strains No. 2-193 and No. 9731 are antigenically unlike.

Absorption experiments were carried out to determine whether agglutination of heterologous organisms by antisera to Assis type II and No. 2-193 is due to antigenic components common to these strains. Anti-Assis type II serum and anti-No. 2-193 serum in dilutions of 1:20 were absorbed with a suspension of strain No. 2-193 in one experiment and a suspension of Assis type II in another. The suspensions were obtained by harvesting the growth on plain agar (Kolle flasks) in 0.4% formaldehyde-saline solution. After centrifu-

TABLE II.
Agglutinin Titer of Native and Absorbed *S. alkalescens* Sera.

S. alkalescens strains	Anti-Assis type II serum			Anti-No. 2-193 serum		
	Native	Abs. with No. 2-193	Abs. with Assis type II	Native	Abs. with No. 2-193	Abs. with Assis type II
Assis type II	*1:4000*	*1:4000*	<1:40	1:200	<1:40	<1:40
No. 2-193	1:400	<1:40	<1:40	*1:4000*	<1:40	*1:4000*
No. 9731	1:40	1:40	1:40	<1:40	1:40	1:40
No. 1-2 (type I)	1:40	1:40	1:40	<1:40	<1:40	<1:40

gation the sera were added to the sediments. The tubes were shaken thoroughly and incubated at 55°C for 4 hours. Unabsorbed serum in like dilution was treated in the same manner. The tubes were centrifuged and absorption was repeated. Then the supernates as well as the native sera were tested for the presence of agglutinins against the 4 strains under investigation. The experiment was carried out in duplicate. The results are summarized in Table II.

The conclusion may be drawn that the hitherto undescribed strain No. 9731 shares no demonstrable antigenic components with Assis type II and strain No. 2-193. On the other hand, strain No. 2-193 contains a small antigenic fraction in common with Assis type II; its major antigenic components, however, differ from the major antigens of Assis type II. Thus, the 4 strains represent 4 different antigenic types.

The conclusion that strains No. 2-193 and No. 9731 as well as Assis type II differ in antigenic structure from *S. alkalescens* type I is further substantiated by the following observation. Fifteen strains of *S. alkalescens* type I, available at this laboratory, were tested with antisera to *S. alkalescens* types I and II as well as strains No. 2-193 and No. 9731. It was observed that all 15 strains were strongly agglutinated to high titer by anti-type I serum, but not at all or only to a small fraction of the titers of the antisera against Assis type II, No. 2-193, and No. 9731.

It is well known that *S. alkalescens* type I may be agglutinated by certain anti-*S. paradysenteriæ* sera. It seemed of interest to determine, therefore, what relationship, if any, strains No. 9731 and No. 2-193 have to paradysentery bacilli. To this end the following experiment was carried out. Various anti-*Shigella* sera, obtained from Lederle Laboratories through the courtesy of Dr. A. J. Weil, were used. They included sera against the following species and types: (1) *S. dysenteriæ* (Shiga), (2) *S. sonnei,* (3) *S. ambigua* (Schmitz), (4) *S. alkalescens,* (5) *S. paradysenteriæ* (polyvalent), and (6 to 19) Flexner types I to XIV representing V, W, X, Y, Z of Andrews and Inman as well as Boyd's types 103, P119, 88 (Newcastle), 170, P288, D1, D19, P143 and 274. One drop of these diagnostic reagents was mixed on a slide with one drop of the bacterial suspension previously heated at 100°C for 30 minutes. Agglutination was read with the naked eye. The following results were obtained: *S. alkalescens* type I (No. 1-2) was agglutinated only by anti-*S. alkalescens* serum. The strains No. 9731 and Assis type II showed a trace of clumping with serum against Flexner type XIII, but none with any of the other sera. Strain No. 2-193 was strongly agglutinated by anti-type XIII serum and not at all by the others. Dr. A. J. Weil also observed agglutination of this strain by anti-type XIII serum. In this connection mention should be made of the observation of Boyd[3] to the effect that *S. alkalescens* and Flexner type P274 cross-agglutinate, although they fail to remove the heterologous agglutinins on absorption. Further studies are necessary to determine the antigenic relationship of *S. alkalescens* No. 2-193 and Flexner type XIII.

The antigenic relationship of the hitherto undescribed types of *S. alkalescens* to the typhoid-paratyphoid group was also studied. Suspensions of *E. typhosa, S. paratyphi, S. enteritidis,* and *S. typhi murium* were tested with the 4 different anti-*S. alkalescens* sera.

[3] Boyd, J. S. K., *J. Hygiene,* 1938, **38**, 477.

It was found that neither of the antisera against types I and II and strains No. 9731 and No. 2-193 agglutinated these organisms to more than a very small fraction (1% or less) of the titers. Likewise, the suspensions of the *S. alkalescens* strains were not agglutinated or agglutinated only to a fraction of the titers of antisera against the typhoid-paratyphoid organisms. It is evident, therefore, that the 4 types of *S. alkalescens* do not share any major antigens with the typhoid-paratyphoid organisms studied.

Summary and Conclusions. Two hitherto undescribed antigenic types of *S. alkalescens* have been reported. The representative strains have the morphological, cultural, and biochemical characteristics of *S. alkalescens* type I and do not share major antigenic components either with each other or with types I and II. One of the strains was isolated by Captain Ewing, the other at this laboratory. At the present time, therefore, *S. alkalescens* comprises 3 antigenic types, namely, type I composed of 4 sub-types, and the 2 new types described here. It is doubtful whether type II of Assis will ultimately be classified as *S. alkalescens.*

Sincere thanks are due to Captain William H. Ewing, Sanitary Corps, United States Army, to Dr. Arlindo de Assis, Rio de Janeiro, Brazil, and to Dr. A. J. Weil, Lederle Laboratories, Pearl River, New York, for material used in this investigation. Major Kingston S. Wilcox, Sanitary Corps, Chief, Division of Bacteriology, kindly gave permission to publish the data obtained on the strain isolated by Captain Ewing.

14755

Effect of Lowered Blood Supply and of Glucose-1-Phosphate on Healing of Bone Fractures.*

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Promotion of bone fracture healing is a serious problem at all times in the advanced age group of the population, because, with advancing age, there is an increased susceptibility to fractures, and difficulty and delay in their healing is a fairly common occurrence. The study of therapeutic measures to promote fracture healing in older individuals has been retarded up to now by lack of a method of producing a delay in the union of fractures comparable to that found in old age.

In this communication there is described a simple experimental procedure which yields a long delay in the healing of bone fractures. The technic consists simply of ligaturing the femoral artery of a limb and thereby reducing the blood supply to the affected bone. By this technic it is hoped that therapeutic measures can be tested which eventually will prove to be of benefit in the treatment of delayed union in the aged. The results obtained on rats by this technic will be presented below.

The high alkaline phosphatase activity of calcifying bone has attracted the attention of investigators since the pioneer work of Robison. Robison's theory[1] that the phosphatase acted to raise the product of calcium and phosphate ions by liberating inorganic phosphate from organo-phosphates in the blood faced the difficulty that the acid-soluble organic ester content of the blood plasma is small. It has been observed that glycogen increases in hypertrophic cartilage prior to ossification and disappears during ossification (Harris,[2] Glock[3]). The presence of the en-

* Aided by grants from the Nutrition Foundation, Inc., and the Christine Breon Fund for Medical Research.

1 Robison, R., *The Significance of Phosphate Esters in Metabolism,* 1932, New York Univ. Press, N.Y.

2 Harris, H. A., *Nature,* 1932, **130,** 996.

3 Glock, G. E., *J. Physiol.,* 1940, **98,** 1.

zyme phosphorylase in hypertrophic cartilage has been demonstrated by Gutman and Gutman.[4] These observations lend themselves to a theory of bone calcification which is represented by the equations given below.

$$\text{Glycogen} + \text{phosphate} \xrightarrow{\text{phosphorylase}} \text{Glucose-1-phosphate}$$

$$\text{Glucose-1-phosphate} \xrightarrow{\text{phospho-glucomutase}} \text{Glucose-6-phosphate}$$

$$\left.\begin{matrix}\text{Glucose-1-phosphate}\\ \text{Glucose-6-phosphate}\end{matrix}\right\} \xrightarrow{\text{bone phosphatase}} \text{Glucose} + \text{phosphate}$$

The above reactions show that bone phosphatase would, as suggested by the theory of Robison, liberate inorganic phosphate and tend to cause a supersaturation of the bone salts. However, according to the above equations, the substrate for the action of the phosphatase is formed in the ossifying cartilage directly and is not brought to the bone by the circulation.

Although bone phosphatase can hydrolyze either of the glucose phosphate esters shown above, not only they, but some further product in the phosphate cycle of carbohydrate breakdown could equally well serve as the substrate for bone phosphatase activity. In the mechanism of the regulation of the blood glucose level by the liver it is postulated that glucose-6-phosphate is the glycogen product which is hydrolyzed by liver phosphatase to glucose and inorganic phosphate (Ostern and Holmes,[5] Cori and Cori[6]).

A corollary which follows from the theory advanced above, is that administration of the proper organo-phosphate should promote bone fracture healing. The effect of β-glycerol phosphate on fracture healing has been tested by Armstrong, Sperling, and Letow,[7] who believe they observed some favorable effect. However, unfortunately, the experiments of the above authors were not controlled by tests of the effect of the administration of an equivalent dose of inorganic phosphate.

In the present investigation preliminary tests of the effect of glucose-1-phosphate (Cori ester) on bone fracture healing have been carried out. The ester was synthesized with phosphate labeled with P^{32} so that the fate of its contained phosphate could be followed by the tracer technic. Negative results were obtained when the ester was injected subcutaneously. It is planned to continue the tests on the therapeutic effects of this and of other organo-phosphates.

Experimental Methods and Results. 1. *Lowered Blood Supply and Fracture Healing.* The left femoral artery was exposed and ligatured lightly so as to diminish but not completely stop the blood flow. While under the anesthetic, both the right and left fibula were fractured with the special forceps designed by Hertz.[8] The rats were sacrificed at intervals of 6, 12, 13, 18, 24, and 30 days after the operation. Each animal was given an intraperitoneal injection of 0.2 mg Sr^{89} in physiological saline solution 48 hours before being sacrificed. The fibulæ were then dissected out and tested for Sr^{89} activity and breaking strength as described elsewhere (Copp and Greenberg[9]). The results are summarized in Table I. It will be observed from the table that the reduction in the rate of bone healing caused by the lowered blood supply is indeed striking.

2. *Effect of Glucose-1-Phosphate on Fracture Healing.* The glucose-1-phosphate was synthesized by the action of the enzyme phosphorylase on glycogen and a solution of inorganic phosphate containing P^{32} (McCready and Hassid[10]). The tests were carried out on rats with the left femoral artery ligatured as described above. From the fifth day after fracture of the fibulæ, the animals were in-

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[5] Ostern P., and Holmes, E., *Nature,* 1939, **144**, 34.

[6] Cori, G. T., and Cori, C. F., PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 337.

[7] Armstrong, W. D., Sperling, L., and Litow, S., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 169.

[8] Hertz, J., *Acta pathol. microbiol.* Scand., 1936, Vol. 26-30, Supplement.

[9] Copp, D. H., and Greenberg, D. M., *J. Nutrition,* in press.

[10] McCready, R. M., and Hassid, W. Z., *J. Am. Chem. Soc.,* 1944, **66**, 560.

TABLE I.
Effect of Lowered Blood Flow on the Rate of Healing of Bone Fractures.

	Ligatured			Control		
Time days	No. rats	Sr^{89} uptake % of dose $\times 10^4$	Breaking strength, g	No. rats	Sr^{89} uptake % of dose $\times 10^4$	Breaking strength, g
6	7	3.73 ± 0.50	0	7	5.36 ± 0.66	0
12	7	6.85 ± 1.00	56 ± 40	7	12.78 ± 0.70	420 ± 100
13	4	7.80 ± 0.80	70 ± 35	4	16.00 ± 1.80	615 ± 95
18	7	9.30 ± 1.54	81 ± 36	7	25.35 ± 3.22	686 ± 200
24	7	8.86 ± 2.64	93 ± 55	7	14.80 ± 2.64	593 ± 150
30	4	7.26 ± 0.40	270 ± 100	4	9.27 ± 0.40	890 ± 20

Measure of variability is mean deviation.

TABLE II.
Effect of Subcutaneous Administration of Glucose-1-Phosphate on Bone Fracture Healing.

	Ligatured		Not ligatured	
No. rats	P^{32} uptake, % of dose	Breaking strength, g	P^{32} uptake, % of dose	Breaking strength, g
		Glucose-1-Phosphate.		
5	1.13 ± 0.17	50 ± 40	1.69 ± 0.42	265 ± 40
		Inorganic Phosphate.		
4	1.32 ± 0.19	30 ± 40	2.40 ± 0.56	270 ± 60

Measure of variability is mean deviation.

jected twice daily subcutaneously with 1 ml of solution containing 0.1 g of the potassium salt of the Cori ester for a period of 5 days. Control animals were similarly injected with 0.5 ml solutions containing an equivalent amount of inorganic phosphate. On the eleventh day the animals were sacrificed and the fibulas tested. The results are given in Table II.

In these experiments there was no observable difference in effect between the glucose-1-phosphate and the inorganic phosphate. Neither reagent noticeably accelerated the healing of the bone with the reduced blood supply. However, there is no assurance that the intact ester reached the fractured bone. Prior hydrolysis by phosphatase may have occurred. More direct means of perfusing the bone with the ester will have to be carried out to make the results conclusive.

Summary. 1. A long delay in bone fracture healing can be produced by reducing the blood supply to the affected bone. This technic is suggested for the study of therapeutic measures to promote the healing of delayed union of bone in the aged. 2. Glucose-1-phosphate, when administered by subcutaneous injection, had no favorable effect on the healing of bone fractures.

14756 P

Experimental Production of Hepatitis by Feeding Icterogenic Materials.*

WALTER P. HAVENS, JR., ROBERT WARD, V. A. DRILL, AND JOHN R. PAUL. (Introduced by F. G. Blake.)

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Although *infectious hepatitis* has become a military problem of great importance,[1–16] its natural mode of transmission is obscure.

Reports by MacCallum and Bradley[17] and perhaps others[18,19,20] already indicate that hepatitis can be acquired by man following ingestion or nasal instillation of the infectious agent in feces, serum, or possibly nasal washings and duodenal drainage. The present report describes 5 definite examples of clinical infectious hepatitis induced by feeding material containing an icterogenic agent in feces and serum.

"Infectious" materials, listed in Table I, employed here were obtained in 1943 from American and British soldiers in Africa and Sicily. Sera and fresh stools were kept at dry-ice box temperature for 8 months; dehydrated Seitz

TABLE I.
Source of Material.

Material	Material obtained from: Inoculated case	Material obtained from: Natural case
Serum P	*	
" No. 1	—35	
" 2	—21	
" 3	—42	
" 4	— 7	
" 5	+ 1	
" 6	+ 1	
" 7		—6
Stool B		+8
Dehydrated filtrates of stool and urine extracts V.R.†	?	?

* Serum P was pooled from 11 men in the first 24 hours of sandfly fever. It was later found to be icterogenic. This pool was prepared by Maj. A. B. Sabin, M.C., and was used in studies reported by Paul *et al.*[21] Serum P was inactivated at 56°C for 30 minutes before intracutaneous inoculation.

Sera Nos. 1-6 were passages from Serum P.

† These filtrates were prepared and dried by Maj. C. E. van Rooyen, R.A.M.C., from patients in various stages of infectious hepatitis at the 15th Scottish Hospital, Middle East Forces.

(—) indicates days before onset of icterus.

(+) indicates days after the onset of jaundice.

* Representing work done under the Neurotropic Virus Disease Commission of the Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the Army, Preventive Medicine Service, Office of the Surgeon General, U. S. Army, Washington, D.C.

Acknowledgment is made of the assistance and cooperation of the following agencies: Selective Service, Camp Operations Division; Civilian Public Service Unit No. 68; Civilian Public Service Unit No. 81; the Norwich State Hospital, Norwich, Conn.; and the Connecticut State Hospital, Middletown, Conn.

1 Senevet, G., Moutrier, P., Gros, H., Alcay, L., and Bougarel, R., *Arch. Inst. Pasteur d'Algerie*, 1941, **19**, 47.

2 Dietrich, S., *Deutsche med. Wchnschr.*, 1942, **68**, 5.

3 Gutzeit, K., *München med. Wchnschr.*, 1942, **89**, 161, 185.

4 Siegmund, H., *ibid.*, 1942, **89**, 463.

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6 van Rooyen, C. E., and Gordon, I., *J. Roy. Army M. Corps*, 1942, **79**, 213.

7 di Benedetto, J., *La Settimanne Medico*, 1942, **30**, No. 50.

8 Cameron, J. D. S., *Quart. J. Med. N. S.*, 1943, **12**, 139.

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10 (Editorial), *J. A. M. A.*, 1943, **123**, 636.

11 Dixon, H. B. F., *J. Roy. Army M. Corps*, 1944, **82**, 44.

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13 Gear, H. S., *Brit. M. J.*, 1944, **1**, 383.

14 *Bull. U. S. Army M. Dept.*, 1944, No. 76, 26.

15 Witts, L. J., *Brit. M. J.*, 1944, **1**, 739.

16 Havens, W. P., Jr., *J. A. M. A.*, 1944, **126**, 17.

17 MacCallum, F. O., and Bradley, W. H., *Lancet*, 1944, **2**, 228.

18 Voegt, H., *Abstr., Bull. Hyg.*, 1942, **17**, 331.

19 Findlay, G. M., and Martin, N. H., *Lancet*, 1943, **1**, 678.

20 MacCallum, F. O., and Bauer, D. J., *ibid.*, 1944, **1**, 622.

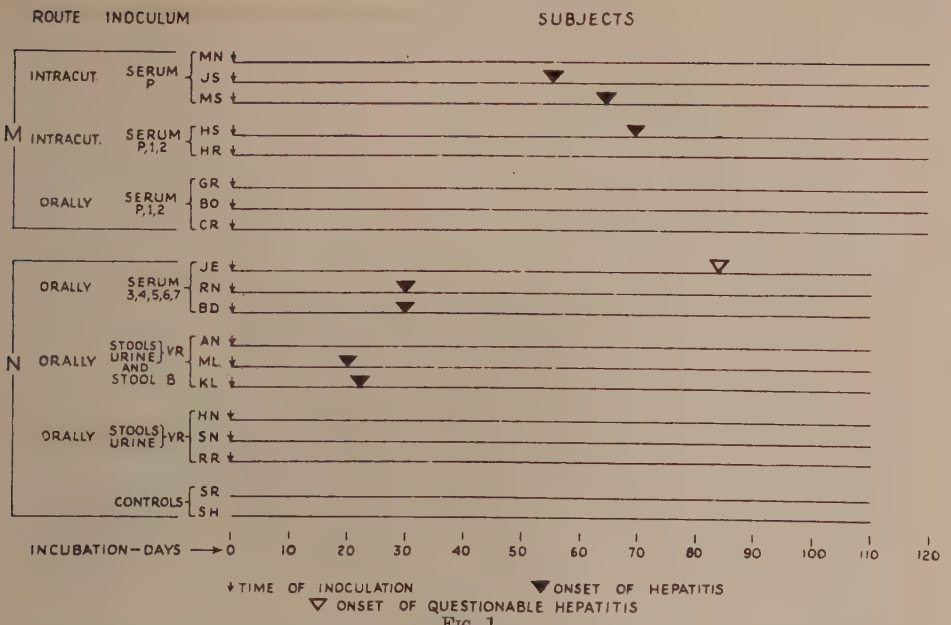

FIG. 1.

Illustration of results of administration of icterogenic material to 2 groups of volunteers at institutions M and N respectively, each subject being represented by a horizontal line. The inoculum which these patients received is indicated by numbers in the left hand column of Table I.

filtrates of urine and stool extracts at room temperature or ice box temperature for 4 months. Both sera and the dried filtrates of urine and stools were sterile on culture before use. Stool B contained no pathogenic bacteria.

The experiment consisted in administering these materials by different routes to 2 groups of human volunteers located in 2 different institutions. For purposes of clarity certain points are illustrated in Fig. 1.

At Institution M, 8 men have been observed for 125 days. Five of them were inoculated *intracutaneously* with serum known by previous tests[21] to contain icterogenic agent. Of these, 3 contracted infectious hepatitis with moderately severe clinical jaundice in 2, and mild jaundice in the third after 56, 65, and 70 days respectively. The remaining 3 volunteers of this first group were *fed* (and given intranasally) icterogenic serum; to date they have remained well. No other cases of infectious hepatitis have occurred in this institution (M) during the period of observation.

At Institution N, 11 men have been observed for 110 days. They were divided into 3 subgroups of 3 men each, with 2 men kept as controls. One subgroup of 3 was *fed* (and given *intranasally*) sera suspected to contain icterogenic agent. Of these, 2 contracted infectious hepatitis with severe clinical jaundice 30 days after feeding and a third developed mild subicteric hepatitis after 84 days. From the blood of one of these men an organism of the salmonella group (*sal. cholera suis*) was cultured during early stages of jaundice. His stool contained no pathogenic bacteria. See Fig. 2.

Another subgroup of 3 men was *fed* urine and stool extracts V.R. *plus* Stool B. Of these men, 2 contracted hepatitis 20 and 22 days respectively following feeding. From the blood of one man *Salmonella cholera suis* was recovered. His stool contained no pathogens.

The third subgroup of 3 men was *fed* urine and stool extracts V.R. All of these patients have remained well.

[21] Paul, J. R., Havens, W. P., Jr., Philip, C. B., and Sabin, A. B., *J. A. M. A.*, to be published.

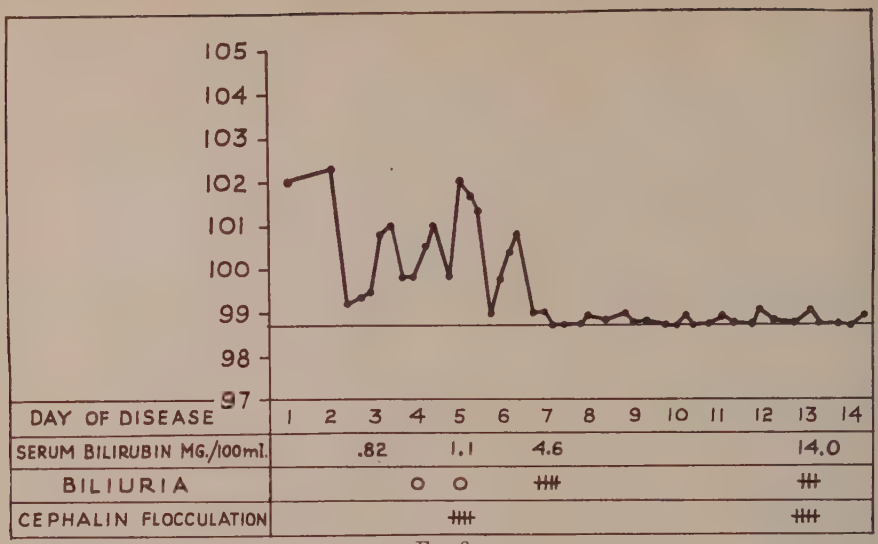

FIG. 2.

Clinical course of volunteer BD. The 7-day period of pre-icteric fever is evident. Rectal temperatures are recorded.

The 2 controls, living in close proximity to the other members of the experimental group, have also remained well.

It is to be noted that *Sal. cholera suis* was isolated from the blood of 2 of the 5 experimental cases induced by feeding icterogenic agent. We have presumed that this organism is a secondary invader, for salmonella infections had been present in Institution N, where these men were quartered, prior to the feeding experiments. It recalls the association of salmonella infections and epidemic hepatitis previously described by French Army workers in 1916,[22] and subsequently discussed by others.[23,24]

One case of acute hepatitis with moderately severe jaundice appeared among the personnel of Institution N, 51 days after the beginning of the experiments there and 31 days after the appearance of the first case among the human volunteers fed icterogenic material. This patient had worked at night at the same desk occupied originally by the first patient to contract the disease experimentally. Although this patient with the "naturally acquired" disease was away on trips before the onset of his disease it is difficult to regard him as an imported case of hepatitis. It is as yet too early to postulate the exact significance of these events.

Summary. An experiment on the transmission of infectious hepatitis has been carried out among 19 volunteers at 2 institutions. At one of the institutions 3 different samples of serum containing the icterogenic agent were inoculated intracutaneously into 5 human subjects, and the disease was produced in 3 after incubation periods of 56 to 70 (avg 64) days.

At another institution other samples of serum and also specimens of feces (and urine) were fed to 9 human subjects, and the disease was produced in 5 after an incubation period of 20 to 84 (avg 37) days by both of these materials.

One case of "naturally acquired" hepatitis has appeared among the institutional personnel 51 days after the beginning of the experiment and 31 days after the appearance of the first experimental case.

22 Sarrailhé, A., and Clunet, J., *Bull. et Mem. Soc. de hôp.*, Paris, 1916, 3s, **40**, 563.

23 Wilcox, W. H., *Brit. M. J., London*, 1919, **1**, 639.

24 Klemperer, P., Killiam, J. A., and Heyd, C. G., *Arch. Pathol. and Lab. Med.*, 1926, **2**, 631.

14757

Efficiency of Muscle Contraction Following Tetanic Stimulation.

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The enhanced contraction of a muscle to single test shocks after tetanic stimulation of its motor nerve was first reported by Schiff.[1] Waller[2] observed an increase in the action potentials of a nerve subjected to tetanic stimulation. Hofmann[3] studied post-tetanic enhancement in muscle. Since the more recent work of Guttman, Horton, and Wilber[4] many publications have appeared on this subject. The phenomenon has been demonstrated in nerve, muscle, and ganglion. It occurs in muscle which has been either directly or indirectly stimulated, and in denervated and curarized muscle. The present study was initiated with the object of determining whether post-tetanic facilitation in striated muscle is related to an improvement in the efficiency of contraction.

Methods. The apparatus and technic employed was similar to that described by Cattell and Feit.[5] A double sartorius preparation from the frog (*Rana pipiens*) was set up on a thermopile and the response to maximal induced break shocks was photographed by means of an optically recording isometric lever. The thermopile-galvanometer system had a sensitivity such that heat resulting from a single twitch of the muscles produced a deflection of from 150-300 mm on a scale placed three meters from the mirror of a moving coil galvanometer (Zernicke Zc). The maximal deflection was taken to be proportional to the initial heat production. Changes in the efficiency of contraction were determined by changes in the ratio of the tension developed to the initial heat in individual twitches. The moist chamber was immersed in a water bath, the temperature of which was maintained at 22°C.

In all instances the muscles were immersed in Ringer's solution (NaCl 0.675%, $CaCl_2$ 0.020%, KCl 0.015%, phosphates 0.01% to give a pH value of 7.2) for at least one hour before making observations. The Ringer's solution was removed and the muscles were allowed to remain in moist oxygen for about half an hour before starting the experiment. This procedure tends to promote a uniformity of response and a steady galvanometer reading. The experiments were all performed in an atmosphere of moist oxygen. Maximal break shocks and a tetanizing current, applied for various lengths of time, which varied from 90 shocks per second to lower frequencies, were employed. The source of stimulation was either the secondary of an inductorium or a thyratron stimulator.

In all of the 24 experiments performed test shocks were given to determine the tension developed (T) and the initial heat production (H), and from these T/H values were calculated for individual twitches. The muscle was then subjected to a brief tetanus of a few seconds duration following which test shocks were delivered at various intervals. Heat measurements could not be made until about 3 minutes after each period of tetanus because of the rapid heating during tetanus and subsequent cooling. During cooling the galvanometer at first drifted rapidly and then more slowly. True stability of the galvanometer was generally not reached for at least 10 minutes after a tetanus but it was possible to correct for the slower type of decay. The drift during the 4-second period required for

[1] Schiff, J. M., *Muskel und Nervenphysiologic Lahr*, 1858, 189.

[2] Waller, A. D., *Phil. Trans. Roy. Soc., B*, 1897, **188**, 1.

[3] Hofmann, F. B., *Arch. f. d. ges. Physiol.*, 1903, **93**, 186; 1903, **95**, 484; 1904, **103**, 291.

[4] Guttman, S. A., Horton, R. G., and Wilber, D. T., Proc. Soc. Exp. Biol. and Med., 1936, **34**, 219; *Am. J. Physiol.*, 1937, **119**, 463; Horton, R. G., Wilber, D. T., and Guttman, S. A., *Am. J. Physiol.*, 1937, **119**, 338.

[5] Cattell, McK., and Feit, H., *J. Physiol.*, 1937, **91**, 259.

the galvanometer to reach its maximum deflection resulting from the superimposed heat from the twitch was determined by recording the change during 6 five-second periods before a test shock and exterpolating to obtain the correction for the position at the end of the 4-second period. It is unfortunate that heat values could not be obtained immediately, since this fact prevented the study of the first part of the post-tetanic enhancement curve.

Results. Fig. 1 is a graph of a typical experiment in which the tetanus (about 50 per second for 30 seconds) produced enhancement. The twitch tension directly after the first tetanus was 29% greater than that developed prior to the tetanus. The heat production in the twitch 3 minutes after the tetanus, when the tension had fallen to about 12% above the pre-tetanic values, was 21% greater and the T/H ratio 10% below the pre-tetanic value. In the course of 25 minutes tension values gradually approached those of the pre-tetanic twitches. Heat values dropped rapidly reaching a low of 2.5% below the normal in 19 minutes. Correspondingly, the efficiency (T/H) rose from the initial 10% below normal to a peak of 10% above normal in 6 minutes after the tetanus and then gradually returned to the control value. Following the second period of tetanus an almost identical pattern was observed save that the low T/H values directly after the tetanus were not observed.

The result illustrated above is typical of the findings observed in post-tetanic enhancement in 14 experiments. From 4 to 6 minutes after tetanus the T/H values were depressed in the average 8.5% but then increased, generally within 1 to 3 minutes, to a peak average of 6% above the pre-tetanic level. Return of T/H ratios to the original value generally occurred in from 20 to 30 minutes.

Fig. 2 is a graph showing the results in an experiment in which the muscle exhibited post-tetanic depression. Three series of tetanizing shocks were administered. The duration of the first was 10 seconds, the next 3 seconds, and the last 10 seconds. Following the first period of tetanus the twitch tension was 48% and the heat 41.6% of the pre-tetanic value, resulting in a T/H ratio 15% above the pre-tetanic level. These indices returned towards their respective pre-tetanic values as indicated in the graph. The changes following the two succeeding periods of tetanus were not as marked but followed the same pattern. The results described for this preparation are typical of 10 experiments in which post-tetanic depression was observed, *i.e.*, the depressed response shows a slightly improved T/H ratio, which gradually returns to the control value over a period of from 5 to 10 minutes. This is in line with the effect of activity on the efficiency of contraction reported by Cattell and Feit.

Discussion. From these data it is apparent

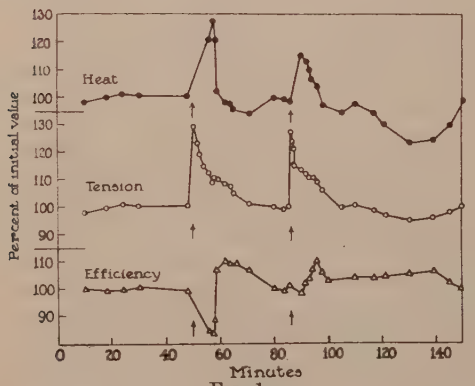

FIG. 1.

The effect of brief periods of tetanus (at arrows) on the twitch tension initial heat and efficiency of contraction in a muscle showing post-tetanic enhancement.

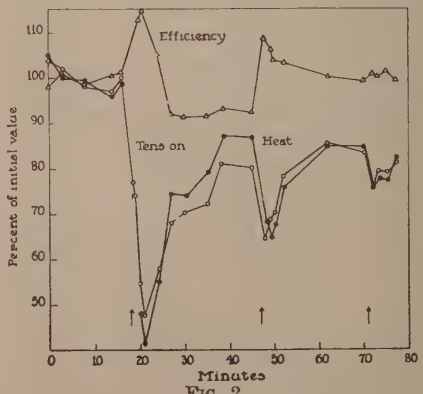

FIG. 2.

The effect of brief periods of tetanus (at arrows) on the twitch tension initial heat and efficiency of contraction in a muscle showing post-tetanic depression.

that enhancement of twitch tension produces a certain pattern of change in efficiency. The twitches directly following a tetanus are greatest and the decay has a linear relationship. While twitch responses occurring shortly after a period of tetanus show characteristic changes in efficiency, they are not large in comparison with the changes in tension. Enhancement, as measured by twitch tension, is at first associated with a small decrease in efficiency and then a small increase in efficiency with a return to pre-tetanic levels. On the other hand, when the post-tetanic twitch is depressed, the efficiency is temporarily increased.

Post-tetanic twitch responses may show enhancement or depression with tensions of 50% greater to 50% less than the pre-tetanic values. The initial heat developed with each twitch varies with the tension so that the efficiency of contraction is not markedly altered, and cannot account for the changes in tension.

Summary. 1. The twitch tension and the initial heat developed was determined in frog double sartorius preparations during the period following tetanic stimulation. 2. When enhancement occurs there is at first a small decrease in efficiency, followed by a rise slightly above the original level, with return to the pre-tetanic level, as the enhancement subsides. 3. When post-tetanic depression occurs there is a small increase in efficiency followed by a gradual return to the pre-tetanic value. 4. The efficiency changes are small in comparison with the changes in twitch tension, and thus cannot account for the changes in the mechanical response.

14758

Estimation of the Anticephalin Activity of Whole Blood.

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The demonstration of anticephalin* activity in plasma is rendered difficult by a number of interfering factors. The time and manipulations involved in the separation of the plasma, the addition of anticoagulants and the subsequently required recalcification, the inactivating effect of contact with glass or similar surfaces are among some of the factors that interfere with the accurate testing of this activity in plasma.[1,3] Since anticephalin manifests itself chiefly during the initial stages of coagulation (before the conversion of prothrombin), any imperfections in the collection of blood or in its mixture with the anticoagulant will affect the results. It would seem desirable, therefore, that the activity be estimated without the use of anticoagulants, and as soon as the blood is collected.

As shown elsewhere[4] the response of a given plasma to activation by cephalin, the magnitude of the clot-decelerating effect of incubation of the plasma with cephalin, and the difference between the rate of coagulation of plasma on glass and on paraffin or lusteroid

* The term *cephalin* is used to designate the alcohol-insoluble lipid fraction extracted with ether from acetone-dried human brain.[1] Cephalin suspensions so obtained are obviously not pure. Most indications point to a cephalin as the active fraction of the thromboplastic lipoprotein[2] and it may so be considered until convincing evidence to the contrary is available. The term *anticephalin* is intended to designate that activity of plasma directed against the thromboplastic action of cephalin.

1 Tocantins, L. M., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 94.

2 Quick, A. J., *Hemorrhagic Diseases and the Physiology of Hemostasis*, 1942, p. 68.

3 Tocantins, L. M., *Am. J. Physiol.*, 1943, **139**, 265.

4 Tocantins, L. M., *Am. J. Physiol.*, in press.

surfaces appear to maintain a certain correlation, when other factors (prothrombin, antithrombin) remain equal or under control. When cephalin is added to plasmas with low anticephalin activity, clotting occurs more rapidly than normal, and at about the same rate in glass or lusteroid tubes. Conversely, in the presence of excessive anticephalin activity (*e.g.*, hemophilic plasma), the clot-accelerating effect of cephalin is lessened, and the difference between the rate at which coagulation proceeds in glass as compared with paraffin-coated or lusteroid† tubes is magnified.[4] When dealing with intact whole blood it is manifestly not feasible to estimate anticephalin activity by incubating blood with the lipid. It appears, however, that some dependence may be placed simply on the response of the blood to the cephalin and on a comparison between its rate of coagulation on glass and other surfaces.

Methods. Human venous blood is collected into an oiled syringe observing the especial precautions previously outlined.[3] Since the addition of cephalin must take place at the bedside, as soon as the blood is collected, tubes (13 mm i.d. x 60 mm length, one of pyrex glass, the other of lusteroid or glass coated with paraffin) containing the measured amount of cephalin suspension are held ready immersed in water at 38°C, in a wide neck thermos bottle. One cc of blood is mixed with 0.1 cc of the cephalin suspension and the time required for coagulation measured with a stopwatch. The clotting time of plain blood in the two types of tube is also measured, simultaneously. When citrated as well as intact blood are desired, an assistant is necessary to insure expeditiousness in handling the collection of blood into 2 syringes, the first one holding the anticoagulant (1 part of a 0.129 M solution of trisodium citrate to 9 parts of blood), the second reserved for plain blood.

Plasma anticephalin activity is estimated by noting the delay in the clotting time induced by incubating (38°C) the citrated plasma with cephalin for 20 minutes before recalcification. Both glass and lusteroid tubes have been used. In view of the disturbing effects of contact with glass,[4] the results obtained on lusteroid surfaces are considered to represent the actual extent of this activity. Methods for preparing the cephalin suspension and other technical details are described elsewhere.[1,3,4]

Results. Venous blood to which cephalin is added takes about twice as long to clot in lusteroid as in glass tubes (Table I). The clot delaying effect of incubation of the citrated plasma with cephalin is, however, more than 4 times as great in lusteroid tubes, a fact which has led us to use these or similar tubes almost exclusively in testing anticephalin activity. The prolongation is not due to changes in the plasma brought about by the incubation *per se*. When normal citrated plasma (0.3 cc) was allowed to stand for 20 minutes in a stoppered glass tube at 38°C, its mean clotting time (11 samples) upon recalcification and addition of cephalin was 81.4 seconds in contrast with the mean time of 126.1 seconds when the plasma had been previously incubated with cephalin before recalcification. When lusteroid tubes were employed, the respective times (11 samples) were as follows: Plasma alone, at 38°C for 20 minutes before adding $CaCl_2$ and cephalin = 237 seconds; plasma incubated with cephalin for 20 minutes before recalcification = 393.8 seconds.

The response of a given plasma to activation by cephalin is closely correlated with its anticephalin activity (Fig. 1, Chart 1). Plasmas exhibiting the longest clotting times after the addition of cephalin (*e.g.* hemophilic) are also those best able to reduce the clot accelerating action of the lipid after incubation with it. An even higher positive correlation exists between plasma anticephalin activity (lusteroid tubes) and the cephalin clotting time of blood in a lusteroid tube (Fig. 1, Chart 2). Moreover, the longer is the cephalin clotting time of blood in a lusteroid tube the greater is the difference between that time, and that measured in a glass tube (Fig. 1, Chart 3). This appears to indicate that whatever influences this difference, influences in a similar fashion the clot-accelerating action of cephalin on the blood. Actually the difference between the reaction of the blood to

† Plastic cellulose derivative manufactured by the Lusteroid Co., South Orange, New Jersey.

TABLE I.

Clotting Time in Glass and Lusteroid Tubes of Venous Blood of Normal Young Men (Ages 20-26), with and without Addition of Cephalin. Clotting Time of Normal Plasma with and without Incubation with Cephalin.

	Blood				Plasma			
					Cephalin clotting time‡			
	Clotting time*		Cephalin clotting time†		Glass		Luster.	
	Glass	Lust.	Glass	Lust.	0′ inc.	20′ inc.	0′ inc.	20′ inc.
Mean (sec)	615.6	1953	124.1	244.6	91.5	114.8	194.6	362
Stand. deviation (sec)	80.9	374.6	13.2	38.6	13.3	15.4	41	109
Maximum (sec)	780	2700	163	300	115	158	297	730
Minimum (sec)	480	1320	101	166	72	85	122	202
No. determ.	26	26	26	26	33	33	34	34
No. subjects	19	19	19	19	26	26	26	26

* 1.0 cc venous blood in 13 mm i.d. tubes at 38°C.

† 1.0 cc venous blood + 0.1 cc cephalin, in 13 mm i.d. tubes at 38°C.

‡ 0.3 cc plasma, 0.1 cc cephalin (0′ or 20′ incubation), 0.1 cc 0.074 M $CaCl_2$, in 13 mm i.d. tubes at 38° C.

cephalin in the two tubes has yielded a high correlation with the anticephalin activity of plasma (Fig. 1, Chart 4). On the basis of the above relationships, the cephalin clotting time of blood in lusteroid, paraffin, or similarly coated tubes and the difference between it and that measured in a glass tube may be used with advantage in estimating anticephalin activity. Such relationships, however, apply only when the prothrombin content is near normal, and there is no excess of antithrombin. A naturally occurring excess of antithrombin in blood is rare, but the possible existence of a low prothrombin makes a quantitative determination of this variable necessary. The regression equation correlating cephalin clotting time of blood with plasma anticephalin activity (Fig. 1, Chart 2) must, as further data accumulate, include a correction for the amount of prothrombin in the blood.

The cephalin clotting time of blood is also significantly correlated with the rate of coagulation of plain blood (without cephalin) (Fig. 1, Chart 5). Bloods which respond slowly to cephalin also show the greatest difference in their rate of coagulation between glass and lusteroid tubes (Fig. 1, Chart 6). This may indicate that, unlike certain thromboplastic agents, cephalin acts as the natural clot accelerators of the blood (platelets, leucocytes) and is, like them, vulnerable to their natural antagonists.

In Table II are given examples of these determinations in patients with hemorrhagic disorders. After hemorrhage (subjects 1 and 2) there is hypercoagulability of the blood, shortening of the cephalin clotting time of blood and plasma (especially evident in lusteroid or paraffin coated tubes), diminution in plasma anticephalin activity and reduction or even elimination of the difference in the rate of coagulation of blood and plasma between the two tubes. The relative number of platelets does not seem to influence the behavior of the blood towards the cephalin in the two tubes. The presence of a thrombopenia *per se* (subject 3, not bleeding at the time of observation) likewise leads to no significant changes in the blood or plasma cephalin clotting time. In hemophilics (*e.g.*, subject 4) the blood and plasma respond slowly to cephalin, a wide difference is observed between the rate of coagulation in the two tubes, and the plasma anticephalin activity is high. The clotting times of hemophilic blood with and without addition of cephalin are longer than those of hypoprothrombinemic (subject 5) or heparinized (subject 6) blood. Plasmas separated from hypoprothrombinemic and heparinized blood often display an increase in anticephalin activity, the magnitude of which depends on the degree of hypoprothrombinemia or the amount of heparin present. The exaggeration of anticephalin activity in moderately heparinized blood manifests itself, however, principally when testing

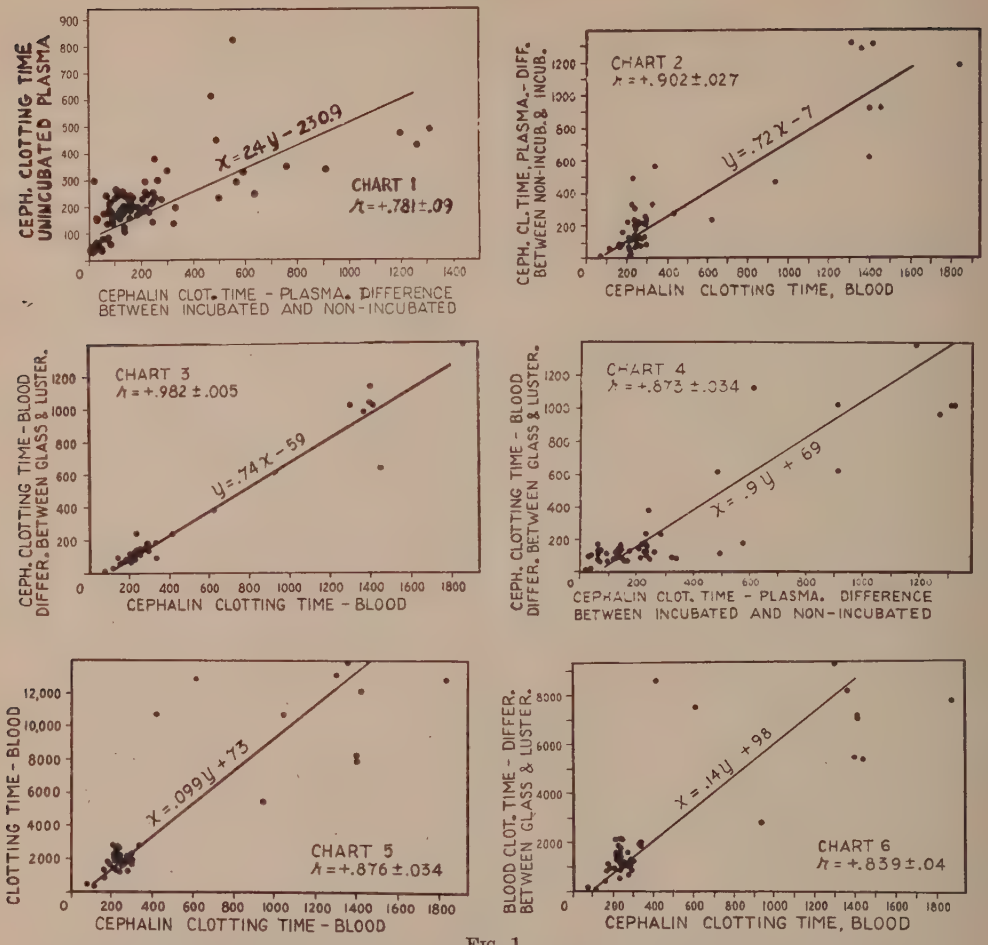

FIG. 1.

Correlations between the rate of coagulation of human blood and plasma in glass and lusteroid tubes, with and without incubation with cephalin. Unless otherwise specified, the clotting times (expressed in seconds) refer to determinations in lusteroid tubes. No determinations included in the above group if the prothrombin was lower than 70% of the normal, or if there was an excess of antithrombin (heparinized blood).

the plasma (Table II) and may in part result from artifacts introduced during the separation, collection, standing, and recalcification of the plasma. An indication, however, that some of the anticephalin effect is usually lost during these steps is obtained by comparing the cephalin clotting times of blood in the 2 tubes with that of the respective plasmas (unincubated) (Tables I and II). While in normal subjects the times on plasma and blood in the same tube are almost alike, in fresh hemophilic blood the cephalin clotting time in the two tubes may be more than twice as long as that of its unincubated plasma. This supplies an additional reason for testing this function in fresh blood, without anticoagulants.

The superimposition of hypoprothrombinemia or heparinemia would naturally exaggerate the anticephalin effect. The need of a prothrombin determination is therefore apparent for the proper estimation of the significance of a prolonged cephalin clotting time of venous blood. This determination may be con-

TABLE II.
The Coagulation Time in Glass and Lusteroid Tubes of Venous Blood with and without Additions of Cephalin, and of Plasma before and after Incubation with Cephalin. Human Subjects with Various Hemorrhagic Disorders.

	Subject											
	1 Post-hemorrhagic, with thrombopenia		2 Post-hemorrhagic, with thrombocytosis		3 Thrombopenic (nonhemorrh.)		4 Hemophilic		5 Hypoprothrombinemic (dicoum.)		6 Heparinized†	
Determination*	Glass	Lust.	Glass	Lust.	Glass	Lust.	Glass	Lust.	Glass	Lust.	Glass	Lust.
Clot. time, blood (sec)	360	480	480	420	540	1440	5100	12900	690	2760	1860	2940
Ceph. clot. time, blood (sec)	75	81	86	112	99	201	830	1449	204	579	220	480
Ceph. clot. time, plasma 0′ incub. (sec)	74	90	71	96	87	192	238	618	92	235	203	792
20′ incub. (sec)	80	109	76	64	100	329	345	2296	157	860	532	>3600
Blood platelets (thous./mm³)	20		510		18		430		180		154	
Plasma prothr. (%)												
1-stage meth.	90		100		90		100		22		95	
2-stage meth.	72		70		108		110		20		90	

* For methods see footnotes under Table I.
† 40,000 units Lederle Heparin intravenously over a period of 48 hours. Man, wt 65 kg.

veniently done at the time the blood is collected by one of the standard bedside methods,[2] or in the plasma by the two-stage method.[5] Naturally occurring increases in the antithrombin content of the blood are seldom encountered, and require no especial technical provision in clinical studies.

Summary. The rate of coagulation of whole blood in paraffin coated or lusteroid tubes after addition of a standard suspension of cephalin, gives a rapid and reliable estimation of the anticephalin activity of the corresponding plasma. The extent of the difference between the rate of coagulation of blood in glass and lusteroid tubes supplies an additional estimate of this activity. The response to the clot accelerating action of cephalin does not seem to be significantly affected by the platelet content of the blood; the presence of hypoprothrombinemia may, however, exaggerate the delay in the response.

[5] Smith, H. P., Warner, E. D., and Brinkhous, K. M., *J. Exp. Med.*, 1937, **66**, 801.

14759

Effect of Penicillin and Patulin on Fowl Pox.*

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The effects of penicillin and patulin upon the infection of fowl pox, contagious epithelioma, of the chorioallantoic membrane of the developing chick embryo have been determined.

* This work was supported by a grant from the Mallinckrodt Chemical Works.

Procedure. Forty-five eggs which had been incubated for 11 days were prepared for inoculation by the technic described by Good-

pasture *et al.*[1] The inoculation was done by gently rubbing a small piece of infected membrane over the center of the chorioallantoic membrane after it had fallen away from the shell. The opening in the shell was closed by sealing a glass cover slip with a petrolatum-paraffin mixture. All 45 eggs were inoculated and divided into 3 groups of 15 each. Group 1 served as the control; 0.25 cc of saline containing 100 Oxford units of penicillin was placed upon the inoculated membrane of each embryo in Group 2 one hour after inoculation and every 24 hours thereafter for 4 days; 0.25 cc of saline containing 0.1 mg of patulin was placed upon the membrane of each embryo in Group 3 one hour after inoculation and every 24 hours thereafter for 4 days. At the end of 5 days the membranes from the live embryos were removed for gross and microscopic study and for subsequent inoculation of 10-day-old chicks; 0.5 cc samples of fluid from immediately under the membrane of 10 embryos receiving penicillin were collected for assay of the penicillin present.

Results. The membranes from all of the control embryos and from all of those receiving penicillin showed the typical lesions of fowl pox on gross as well as on microscopic examination. Ten 10-day-old chicks inoculated with material from the control membranes as well as ten 10-day-old chicks inoculated with material from the penicillin-treated membranes developed typical fowl pox lesions in 3-5 days.

Assay of the embryonic fluid from 10 penicillin-treated embryos showed a concentration of 20 Oxford units per cc, which is approximately twice what one would expect if uniform distribution occurred throughout the embryo and if there was no destruction of the penicillin by the embryo.

Only 5 of the 15 embryos receiving patulin survived the 5-day treatment. The membranes from this group were necrotic in the center but tissue around the margin showed typical inclusion bodies in and hyperplasia of the epithelium.

Summary. Neither penicillin nor patulin have any inhibitory effect upon the infection of fowl pox of the chorioallantoic membrane of the developing chick embryo.

[1] Goodpasture, E. W., Buddingh, G. J., Richardson, L., and Anderson, K., *Am. J. Hygiene*, 1935, **21**, 319.

I am indebted to Dr. M. T. Bush for the penicillin and patulin as well as for the assay of penicillin.

14760

Thiamine Utilization of Rats Maintained on Diets Containing Dextri-Maltose.

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Najjar and Holt[1] maintained human subjects for a period of 18 months on a purified diet similar to rations employed in nutrition studies with rats. The experiment was designed to establish the minimum thiamine requirement of man. Dextri-maltose served as a source of carbohydrate in the ration. The percentage employed was not given, but in all probability it comprised 50 to 60% of the diet. Despite the fact that the authors stated that repeated examination of the ration failed to show any trace of thiamine, the minute quantities of this vitamin required for the maintenance of health of their subjects suggested that (1) the diet carried appreciable thiamine or (2) the particular carbohydrate had a favorable influence on intestinal "synthesis" of thiamine. The latter possibility was investigated by Najjar and Holt who

[1] Najjar, V. A., and Holt, L. E., Jr., *J. A. M. A.*, 1943, **123**, 683.

found that free thiamine was present in the feces of a symptom-free subject (receiving no thiamine) and disappeared following the administration of succinylsulfathiazole. These findings were taken as evidence of bacterial synthesis of thiamine in the intestinal tract. Although a single diet was employed the statement was made that the biosynthesis of thiamine was affected by diet and that dietary factors other than thiamine might explain paradoxes in the incidence of beri-beri.

The experiments herein reported were undertaken in an effort to study the response of rats to thiamine-deficient diets containing dextri-maltose. Two types of diets were employed; the one in which the so-called "heat stable" members of the vitamin B complex were supplied in autoclaved yeast and the other in which these vitamins were fed in crystalline form. The tests were carried out by both the prophylactic and curative methods.

The diets employed were as follows:

	Diet				
	52	53	54	55	20
	g per 100 g				
Casein (Labco)	18	18	18	18	—
Casein (technical)	—	—	—	—	16
Dextri-maltose	53	—	68	—	—
Sucrose	—	53	—	68	—
Cornstarch	—	—	—	—	60
Autoclaved brewer's yeast	15	15	—	—	9
Salts (U.S.P. No. 1)	4	4	4	4	4
Hydrogenated vegetable oil	8	8	8	8	8
Cod liver oil	2	2	2	2	2
Non-autoclaved dried brewer's yeast	—	—	—	—	0.2

Dried brewer's yeast was spread out in layers one-quarter inch deep and autoclaved at 15 lb pressure for 5 hours at its natural pH.

Diets 54 and 55 were supplemented with 2 mg of riboflavin, 1 mg of pyridoxine, 10 mg calcium pantothenate, 10 mg nicotinamide, 5 mg inositol, and 100 mg choline chloride per 100 g. This supplement was considered ample to cover the requirement of growing rats for these factors.

Thiamine Content of Dietary Constituents (Thiochrome Method). The diets and the carbohydrates and autoclaved yeast contained therein were analyzed for thiamine by the thiochrome method employing the procedure of Hennessy.[2] Dextri-maltose (Mead Johnson) was purchased in the open market and as the containers bore no lot numbers it was impossible to tell whether different batches were used. Three samples purchased at bimonthly intervals were analyzed. The thiamine values obtained for the different lots were in good agreement. All samples of dextri-maltose contained about 0.9 γ per g whereas the sucrose was practically devoid of thiamine (0.06 γ per g) (Table I). The autoclaved yeast employed in the diets was free of thiamine. Consequently the diets containing dextri-maltose carried 0.5 γ thiamine per g and those with sucrose only traces.

TABLE I.
Thiamine Content of Dietary Constituents.

	Thiamin (by thiochrome method) μg/g
Dextri-maltose (1)	.92
" (2)	.93
" (3)	.90
Sucrose	.06
Autoclaved yeast	.00
Diet 52 (dextri-maltose autoclaved yeast)	.50
Diet 53 (sucrose autoclaved yeast)	.00
Diet 54 (dextri-maltose crystalline vitamins)	.52
Diet 55 (sucrose crystalline vitamins)	.08

Prophylactic and Curative Studies with rats. In the prophylactic tests female rats were started at weaning on the above diets. Controls were dosed daily with 40 γ of thiamine. The results as shown in Fig. 1 demonstrate that dextri-maltose carries appreciable amounts of biologically available thiamine. The animals receiving the ration containing dextri-maltose and autoclaved yeast (Curve 1) attained maximum weights which were only 20% below those of the controls dosed with optimal thiamine (Curve 2). On the other hand, the rats maintained on the identical diet but with sucrose as the carbohydrate, succumbed to thiamine deficiency after an initial gain of 20 g (Curve 3). The controls receiving thiamine (Curve 4) grew at a satisfactory rate.

[2] Hennessy, D. J., *Cereal Chem. Bull.*, 1942, **2**, 25.

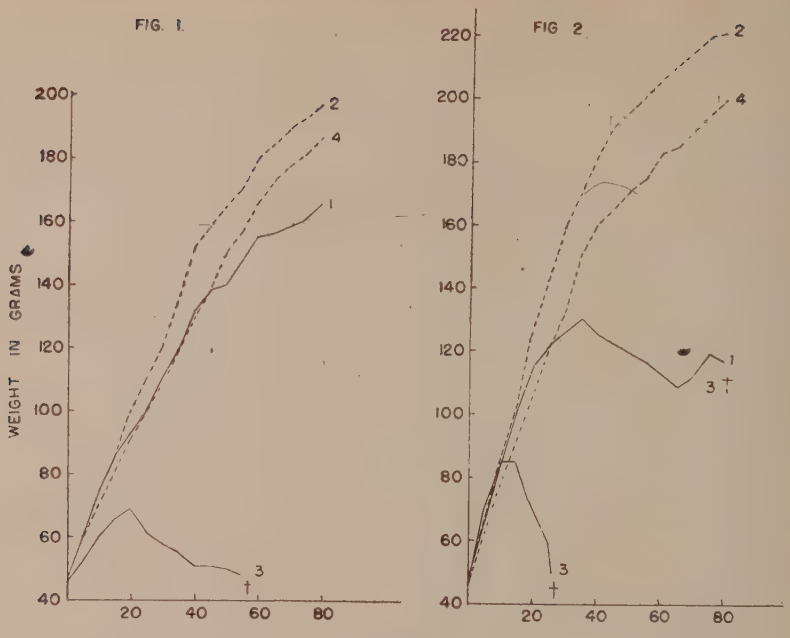

FIG. 1.

Comparison of growth responses with dextri-maltose and sucrose of female rats (5 in each group) maintained on rations containing autoclaved yeast.

Curve 1 Diet 52 (dextri-maltose)
" 2 " 52 + 40 μg thiamine daily
" 3 " 53 (sucrose)
" 4 " 53 + 40 μg thiamine daily

FIG. 2.

Comparison of growth responses with dextri-maltose and sucrose of female rats (5 in each group) maintained on rations containing crystalline vitamins.

Curve 1 Diet 54 (dextri-maltose)
" 2 " 54 + 40 μg thiamine daily
" 3 " 55 (sucrose)
" 4 " 55 + 40 μg thiamine daily

A second prophylactic test comparing dextri-maltose and sucrose was carried out with rats maintained on a diet in which the B vitamins other than thiamine were supplied in crystalline form (Fig. 2). In this experiment the presence of thiamine in dextri-maltose was demonstrated again by the fact that the growth response with dextri-maltose (Curve 1) was superior to that with sucrose (Curve 3). Whereas the rats maintained on the sucrose diet succumbed rapidly (within 26 days) to thiamine deficiency, the animals receiving dextri-maltose reached a plateau in weight during the second month and 3 out of 5 died within the ensuing 60 days. The two remaining rats in this group survived for 120 days at which time the experiment was discontinued. The growth of the controls is shown in Curves 2 and 4.

The curative tests were carried out with two groups of rats depleted of their stores of thiamine by the feeding of a diet (20) employed routinely in the production of thiamine deficiency. At the beginning of the curative test one group had lost 15 to 20 g from their peak weights and the other had declined 20 to 30 g from the maximum. The rats were segregated into groups of like weight losses and weight averages. The controls were allowed to remain on diet 20 and the remaining animals were changed to diets 52 and 54 respectively. As can be seen from Fig. 3, the rats

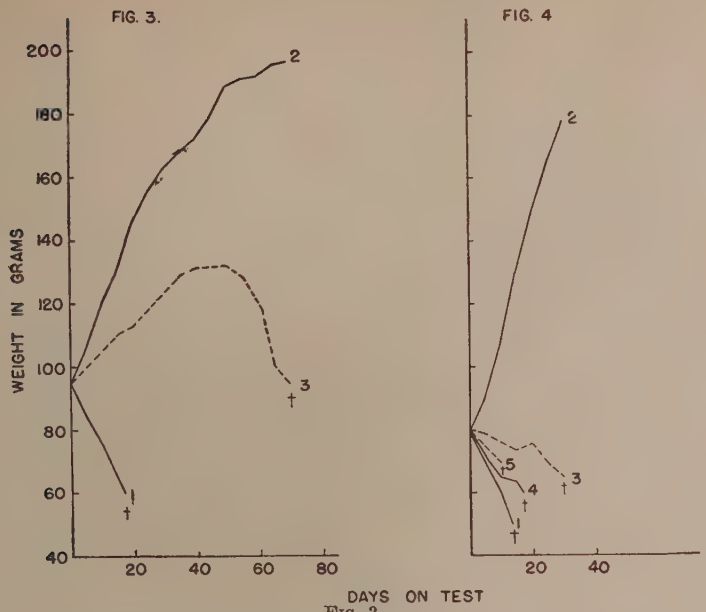

FIG. 3.

Growth responses of thiamine-depleted female rats (5 in each group) to dextri-maltose-containing rations.

Curve 1 Diet 20 (controls)
" 2 " 52 (autoclaved yeast)
" 3 " 54 (crystalline vitamins)

FIG. 4.

Growth responses of thiamine-depleted female rats (5 in each group) to dextri-maltose- or sucrose-containing rations.

Curve 1 Diet 20 (controls)
" 2 " 52 (dextri-maltose, autoclaved yeast)
" 3 " 54 (" crystalline vitamins)
" 4 " 53 (sucrose, autoclaved yeast)
" 5 " 55 " crystalline vitamins)

were rescued from impending polyneuritis and death (Curve 1) by changing from the thiamine-deficient standard diet to the dextri-maltose-containing rations (Curves 2 and 3). However, the animals receiving crystalline vitamins failed to make continuous gains in weight, developed polyneuritis and succumbed within 70 days (Curve 3). The rats receiving autoclaved yeast made continuous gains and were in excellent condition at the termination of the test (Curve 2).

In the second curative test employing rats which had lost 20 to 30 g in weight, the animals were segregated into 5 groups as indicated in Fig. 4. Only the animals receiving the ration containing dextri-maltose and autoclaved yeast (Curve 2) responded with a rapid increment in growth. The average weight decline for the rats receiving the dextri-maltose ration with crystalline supplements (Curve 3) was not as great as for the animals continued on diet 20 (Curve 1) or on the 2 sucrose-containing rations (Curves 4 and 5). Furthermore, the survival time of the rats receiving dextri-maltose was extended from 10 to 20 days beyond that of the other groups. The fact that the weight response did not equal that observed in the previous experiment could be attributed to the debilitated condition of the animals resulting from the longer depletion period.

The superior growth response observed in both prophylactic and curative tests with the diet containing dextri-maltose and autoclaved yeast cannot be ascribed to its thiamine content alone as the ration containing no auto-

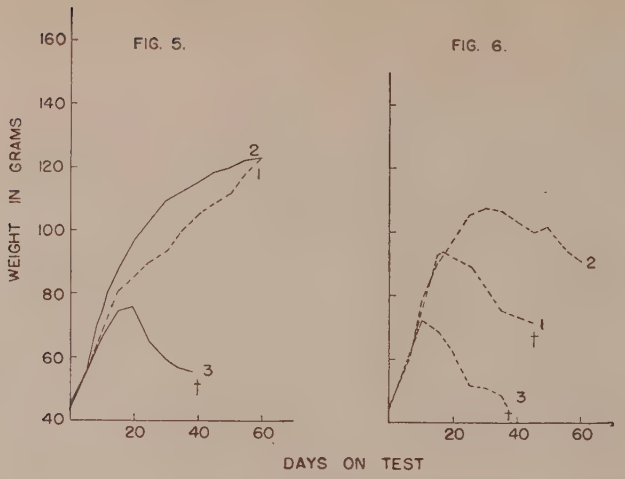

FIG. 5.

Comparison of growth responses with dextri-maltose and sucrose of equal thiamine content of female rats (5 in each group) maintained on rations containing autoclaved yeast.

Curve 1 Diet 53 (sucrose), 0.5 μg thiamine/g added
" 2 " 52 (dextri-maltose)
" 3 " 53 (sucrose)

FIG. 6.

Comparison of growth responses with dextri-maltose and sucrose of equal thiamine content of female rats (5 in each group) maintained on diets containing crystalline vitamins.

Curve 1 Diet 55 (sucrose), 0.5 μg thiamine/g added.
" 2 " 54 (dextri-maltose)
" 3 " 55 (sucrose)

claved yeast gave essentially the same thiamine value by the thiochrome test. Furthermore, the autoclaved yeast *per se* could not explain the differing responses as the same autoclaved yeast was present in the sucrose ration and in diet 20. The possibility existed that degradation products present in the autoclaved yeast were utilized in conjunction with the small quantities of thiamine supplied. In order to test this possibility thiamine was added to the sucrose diet 53 in the concentration present in the dextri-maltose diet 52, namely, 0.5 γ per g. As a check the sucrose ration without the autoclaved yeast (Diet 55) was supplemented in a like manner. The results of these tests are shown in Figs. 5 and 6. As seen from Fig. 5, the rats receiving diet 53 plus 0.5 γ thiamine per g (Curve 1) made weight gains approaching those observed with Diet 52 (Curve 2). Rats receiving diet 53 without added thiamine (Curve 3) succumbed to thiamine deficiency after making maximal weight gains of about 30 g.

On the other hand, rats fed the sucrose diet without autoclaved yeast, but containing 0.5 γ of thiamine per g (Fig. 6, Curve 1), failed to gain at the same rate as did the animals receiving the same intake of thiamine but with dextri-maltose as the source of carbohydrate (Curve 2).

These experiments demonstrate that the growth obtained with small amounts of thiamine (0.5 γ per g) is augmented in the presence of autoclaved yeast which in itself is free of thiamine detectable by the thiochrome method.

Discussion. Dextri-maltose, the carbohydrate employed in the experiments of Najjar and Holt, was found to contain 0.9 γ of thiamine per g as determined by the thiochrome method. The presence of thiamine in dextri-maltose was confirmed by prophylactic and curative tests with rats. However, assays with 2 types of thiamine-low diets, one with autoclaved yeast and the other with the crystalline components comprising the "heat stable" frac-

tion of the vitamin B complex gave divergent results. When thiamine was added to the diet containing sucrose and autoclaved yeast in the same concentration as present in dextri-maltose, the growth response in rats almost equaled that observed with the diet containing dextri-maltose and autoclaved yeast. On the other hand, rats receiving the sucrose-containing ration with crystalline vitamins with added thiamine did not show weight gains comparable to those obtained on the dextri-maltose diet with crystalline vitamins. In this connection it is of interest to note that McIntire and co-workers[3] observed that a sulfite-treated liver extract had a growth stimulating effect in rats maintained on a purified ration sub-optimal in thiamine.

The fact that the growth response to the small amounts of thiamine in dextri-maltose is significantly enhanced by autoclaved yeast led to an investigation of the cleavage products contained in autoclaved yeast and in dextri-maltose. "Resynthesis" experiments with baker's yeast showed that autoclaved yeast carried 4 γ per g of pyrimidine and 7 γ per g of thiazole. This represents about 30% of the original pyrimidine and 50% of the original thiazole moieties of the thiamine present in the yeast before autoclaving. Dextri-maltose contained no pyrimidine, but 0.3 γ of thiazole per g. It appears that these, or other cleavage products, are utilized by the rat in conjunction with small amounts of thiamine. This speculation suggests further research along these lines.

The growth responses obtained in prophylactic and curative tests with diets containing autoclaved yeast are not in agreement with the results obtained by the thiochrome method. From the results obtained by the thiochrome method, identical growth responses were to be expected in rats maintained on diets containing autoclaved yeast or crystalline vitamins. The significant differences observed with the two types of diets in the animal experiments demonstrate that the thiochrome method may not measure all biologically available thiamine.

Summary. Commercial dextri-maltose contains 0.9 γ thiamine per g by the thiochrome assay. Prophylactic and curative tests with rats have demonstrated that the thiamine present in dextri-maltose-containing diets is utilized by the rat. Rats maintained on rations containing autoclaved yeast showed weight increments in excess of those observed with a diet containing the "heat stable" B factors in crystalline form, although both diets contained 0.5 γ thiamine per g by the thiochrome method. Animals receiving diets containing sucrose and autoclaved yeast, but with thiamine added to the same level (0.5 γ per g) present in dextri-maltose, made essentially the same weight gains in prophylactic tests as did rats fed the ration containing dextri-maltose. However, when the autoclaved yeast was replaced by crystalline vitamins, the animals receiving dextri-maltose grew at a rate in excess of that of rats fed sucrose although the thiamine level of the two diets was the same (0.5 γ per g). The results of these experiments indicate that autoclaved yeast apparently carries factor(s) which augment the effect of small amounts of thiamine in the rat. Thus, the thiochrome method may not measure all thiamine which, according to these bioassays, was available to the rat.

[3] McIntire, J. M., Henderson, L. H., Schweigert, B. S., and Elvehjem, C. A., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 98.

14761

Adrenalectomy in Frogs and Toads.*

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A voluminous literature exists on the relationships of the adrenal cortex, carbohydrate metabolism, and electrolyte exchange in muscle. Much of this work has been done on muscles from adrenalectomized amphibians, because isolated surviving preparations from cold-blooded animals are more viable than those from mammals, and there is less chance of introducing complicating variables due to the effect of the adrenal insufficiency syndrome on general metabolism and the nervous and vascular supply to the muscle.

Successful, uncomplicated adrenalectomy in the amphibian is difficult due to the proximity of the uncapsulated diffuse adrenal gland to the vascular supply of the kidney. Damage to this vascular bed may lead to renal failure, edema, asthenia, and death. Most workers therefore have utilized "renal damaged controls" in their studies, and the supposition has been that differences between the control and the experimental groups are due solely to adrenal insufficiency.

More recent workers[1-6] (See for earlier bibliography) have reported variable results regarding survival periods, degree and time of appearance of asthenia, edema, and the relationship of adrenal insufficiency to muscle performance and metabolism.

Although adrenalectomy in amphibians has been worked on since 1891,[7] to our knowledge no one has been able to prove: (1) that his "adrenal insufficient" animals did not have histologically evident large rests of adrenal cortical tissue; (2) that renal failure and uremia did not complicate the syndrome; and (3) that successful replacement therapy was possible by adrenal cortical hormones, followed by a return of insufficiency symptoms which, if not ameliorated, culminate in death after subsequent withdrawal of hormone therapy. The extensive metabolic and work performance studies of muscles from cold-blooded animals in which these tests were not demonstrated may be open to criticism.

Methods and Results. Bullfrogs (*Rana catesbeiana*) and other frogs proved inferior to toads because of their poor wound healing and susceptibility to infections. Large species of toads proved more useful than small species because of the visualization of the adrenal and ease of operation. *Bufo marinus*[8] from Puerto Rico and Bermuda, and *Bufo peltacephalus* from Cuba were investigated next. The Cuban species proved inferior to the *Bufo marinus* for the same reasons given for frogs. The specimens ranged from 5 to 6 inches in body length, thus uniformly larger than most North American species.

The animals were kept in frequently cleaned galvanized iron tanks of 100 gallon capacity, with running water and wooden emergence platforms. They were force-fed every week

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† On leave of absence to Department of Physiology, School of Medicine, University of Southern California, Los Angeles. Other coauthors in armed forces.

1 (A) Fustinoni, O., Dissertation, Buenos Aires, 1938; (B) Fustinoni, O., *Rev. de la soc. Argentina de biol.*, 1938, **14**, 40; (C) Fustinoni, O., *Compt. rend. seances soc. biol.* (Buenos Aires), 1938, **128**, 1137.

2 Maes, J., *C. R. soc. biol.*, 1936, **121**, 848.

3 (A) Angerer, C., and Angerer, H., *Am. J. Physiol.*, 1941, **133**, P197; (B) Angerer, C., *Fed. Proc.*, 1942, **1**, 3.

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with 2-3 No. 000 gelatin capsules containing a balanced small animal ration consisting of 50% dextrinized starch, 30% milk casein, 8% corn oil, 2% cod liver oil, 6% yeast, and 4% USP salt mixture. The toads did well on this diet, maintaining or gaining weight, revealing large fat bodies when killed, and exhibiting great vigor, clasping habits, and general health. Twelve specimens were kept in good health for 3 years by this method.

In adrenalectomizing amphibians, most workers used thermal or diathermy cautery to destroy the adrenals. In the present study several methods were tried in over 100 animals. They were: diathermy cautery, thermal cautery (electro-cautery), freezing cautery, chemical cautery, and various surgical extirpation technics. Of these, only the latter were promising in our hands.

Single or two-stage adrenalectomies by diathermy and thermal cautery destroyed too much renal tissue and blood vessels if sufficient adrenal cortical tissue was destroyed to give insufficiency symptoms. Freezing cautery with ether and solid CO_2-chilled probes did not destroy sufficient tissue. Chemical cautery by saturated trichloracetic acid could not be localized sufficiently to prevent extensive renal damage when enough cortical tissue was destroyed.

Over 60 animals were operated upon using diathermy and thermal cautery and single or two-stage operations, the stages separated by 14-21 days. In one series, unilateral adrenalectomy by diathermy cautery was followed by histological studies to ascertain the extent of renal damage, existence of adrenal cortical rests, and to see if regeneration of renal or adrenal tissues occurred. Autopsy material removed 16-42 days after operation showed marked compensatory hypertrophy of the intact kidney of the unoperated side and a practically non-functional kidney on the operated side, with very few functional glomeruli, vacuolation of renal tubules and extensive lymphocytic infiltration in large areas. This occurred in all cases which did not reveal considerable adrenal cortical rests due to insufficient cautery, permitting indefinite survival.

The trypan blue method of Salmon and Zwemer[9] was employed as a possible test of regeneration of adrenal cortical cells. Six to 10 cc of 0.5% trypan blue were injected into the dorsal lymph sacs over a period of 3-12 days, various times after the operation, and the kidneys studied histologically. There was no indication of regeneration in contrast to the reports for the rat.

The osmic acid method developed by Flexner and Grollman[10] as an indicator of adrenal cortical activity, was useful in thick longitudinal sections of the kidney, for rapid and nearly macroscopic evaluation of the completeness of adrenalectomy, although the stain was poor for renal tissue.

Surgical removal was the only method which yielded successful results. The operative procedure finally evolved was as follows: The toad was anesthetized with Na-pentobarbital, 500 mg per kg in a 0.5% solution in 10% alcohol, thus 1 cc per 100 g of body weight, injected into the dorsal lymph sac or intraperitoneally. After about 10-15 minutes the toad was packed in ice and placed in a refrigerator for 30 minutes or longer, which induced a total hibernation state with no apparent respiratory movements, heart beat, blood flow, or reflexes. The operation was performed with the animal packed in ice to maintain this state.

A one-inch mid-dorsal incision was made through the skin and longissimus dorsi muscle slightly to one side of the transverse spinous processes, in the region of the kidney. The kidney was drawn out of the body cavity through the incision by traction on either the testes or the oviduct. The blood supply to the kidney was cut off by clamping off all the vessels to and from the kidney with delicate hemostatic mosquito forceps. The adrenal tissue was removed from one kidney by cutting the mesothelium covering the kidney and adrenal, around the border of the adrenal, with a sharp-pointed fragment of a razor blade held in forceps. One end of the adrenal was raised with very delicate forceps and the adrenal tissue separated from the kidney with

[9] Salmon, T. N., and Zwemer, R. L., *Anat. Rec.*, 1941, **80**, 421.

[10] Flexner, L. B., and Grollman, A., *Anat. Rec.*, 1939, **75**, 207.

a razor blade fragment or a very sharp, small scalpel. After removal of the adrenal, the hemostats were left clamped for 10-15 minutes or longer to allow clotting of blood exuding from the surface of the cut. With the animal in ice, clotting is retarded. Although its efficacy was not proved, sulfathiazole powder was dusted into the body cavity. The peritoneum and muscle wall were ligated with interrupted stitches of No. 00 gut. More sulfathiazole was dusted on the ligated muscle and the skin was sewn with black surgical thread. Successful procedure necessitated a unilateral adrenalectomy as described, followed no sooner than 5-7 days by unilateral extirpation of the remaining uninjured kidney with its contained adrenal, through a new incision. In performing the nephrectomy, all renal vessels were ligated with surgical silk. It usually required 3 ligatures to ligate all of the genital and renal vessels and the renal portal vein. The mesentery was then cut at the base of the kidney, the kidney removed and the body wall and skin sutured as before.

Two-stage bilateral adrenalectomies were unsuccessful. Death by hemorrhage occurred in all of 7 animals operated upon. Single stage bilateral adrenalectomy also resulted in death. No animals died in which unilateral adrenalectomy was followed later by the unilateral nephrectomy including the remaining adrenal.

Observations of these animals revealed asthenic symptoms which have been described by others.[1B] Examples of such symptoms are, an inability to right when overturned and locomotion by a crawling gait instead of hopping. If the toad was asthenic and edematous, it may have had adrenal insufficiency, uremia, or both. To distinguish these, desoxycorticosterone acetate in oil was injected for several days into the dorsal lymph sac in 3 doses of 0.15 mg 3 times daily. Disappearance of asthenic symptoms after treatment in addition to the ability to void before as well as after treatment indicates that the animal has a functional kidney and that sufficient adrenal cortical tissue was removed to cause an extreme adrenal insufficiency asthenia which probably would be followed by death. Following successful therapy, if the hormone is withheld until subsequent return of the insufficiency symptoms and death, the technic may be considered successful.

Of 12 animals so treated, 4 showed the desired results. The other 8 showed no improvement from the hormone and all died in 3-6 days, average 4. None voided urine, contrary to the 4 successfully operated animals. Autopsy uniformly showed marked edema, empty urinary bladders, vacuolated renal tubules, marked reduction in numbers of functional glomeruli, and extensive lymphocytic infiltration of renal tissue.

The 4 successes were given hormone 7 days. After withholding treatment they died in 3-6 days. Histological examination showed a great reduction in the amount of adrenal cortical tissue present, the remaining cells buried in scattered patches deep in the kidney. Renal damage was apparent, but functional glomeruli and tubules remained in sufficient number to correlate positively with the ability of the animals to void when handled.

It is believed that with experience with this surgical technic and hormone test method, the approximately 30% successes in the small series studied could be increased considerably. The utility of such final test animals in studies of the effect of the adrenal cortical hormones on muscle metabolism, in contrast with mammalian preparations, already has been pointed out in the introduction.

Summary. In a small number of the giant species of toad, *Bufo marinus*, approximately 30% were apparently successfully adrenalectomized without renal failure, by unilateral surgical extirpation of one adrenal followed later by removal of the remaining kidney with its contained adrenal. Hormone maintenance therapy was possible with desoxycorticosterone acetate and, when withheld, was followed by a return of adrenal insufficiency symptoms and death.

14762

Artificial Fever as a Contraceptive.*

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The termination of third day pregnancies in the rabbit by artificial fever was described in an earlier report.[1] The method employed allowed 10 minutes for raising the temperature of the water bath from 100°F to 113°F. Twenty minutes later (30 minutes after immersion) the animals were removed from the bath to have rectal temperatures recorded and then returned to the bath for gradual return to room temperature. The initial rectal temperature of each was known, but the actual time the rectal temperature reached its maximum was not known. Therefore the duration of the period of maximum temperature was unknown.

In the experiments now described the same general procedure was followed except that the 20-minute fever period was counted from the time the anal temperature reached 108°F. This assured that the whole time was spent at the fever temperature, further checked by thermometer readings every 5 minutes.

Each of the 10 does employed had previously borne a normal litter by one of the bucks used in the experimental matings. Sixteen experiments were completed with the 10 does.

The time of acceptance of the buck was recorded for each doe, and, since ovulation is known to take place about 10 hours after mating and fertilization to follow almost immediately,[2] each doe was given a fever treatment between 3 and 12 hours after mating. Litters were produced in 10 of the 16 cases. Examination of the records showed that the 6 not producing litters were all given fever treatment during the 6½ hours following mating while the 10 producing litters received fever treatment 7 to 12 hours after mating. Table I shows for each case the time elapsed since mating, the temperature when the treatment began, and the temperature during the fever period.

Summary. These data indicate that during the first half of the 12-hour period between insemination and fertilization in the rabbit

TABLE I.

Elevations in Temperature of Rabbits During Artificial Fever Treatments and Time Elapsed After Mating in Littering and Nonlittering Does.

Litters Produced.				No Litters Produced.			
Case No.	Time after mating hr min	Normal T°F	Fever T°F	Case No.	Time after mating hr min	Normal T°F	Fever T°F
1	9 40	103.3	110	11	6 30	103.8	110
2	10 17	103.0	109	12	3 22	103.7	108
3	10 22	106.0	110	13	3 40	103.6	109
4	9 18	104.5	109	14	5 46	103.3	110
5	9 20	104.0	110	15	4 30	103.8	109
6	7 30	102.0	109	16	4 50	103.2	109
7	7 25	104.0	109				
8	7 0	102.6	110				
9	12 0	103.5	109				
10	11 0	103.0	109				
Avg	9 20	103.6	109.4	Avg	4 43	103.6	109.2
Avg elevation, 5.8°F.				Avg elevation, 5.6°F.			

* This study was aided by a grant from the Research Council of the University of Missouri.

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1 Cameron, J. A., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 76.

2 Pincus, G., and Enzmann, E. V., *J. Exp. Biol.*, 1932, **9**, 403.

one or both gametes can be fatally injured by 20-minute exposure of the adult female to temperatures of 109°-110°F, involving elevation of general body temperature of 5°-6°F. During the second half of the same period like treatment has no observed effect, but, as previously reported[1] another period of susceptibility to heat is experienced on the 3rd day after mating.

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Chemotherapeutic Properties of Streptomycin.

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The search for a chemotherapeutic agent effective in the treatment of gram-negative bacterial infections recently led to the isolation of streptothricin.[1] This agent was shown to be active *in vitro*[2,3] and *in vivo*[4,5,6] against a variety of gram-negative bacteria. Streptothricin was also shown to be active *in vitro* against the tubercle bacillus.[7,8]

More recently, a second substance was isolated in crude form from a micröorganism called *Actinomycetes griseus*, which was named streptomycin.[9] Like streptothricin, this substance was found to be active against gram-negative bacteria *in vitro* and *in vivo*.[10] The available data suggest that, with the exception of a few microörganisms, the *in vitro* activity of streptomycin appears to be identical with that of streptothricin. Our findings show that a noteworthy difference between these two substances seems to be in the greater activity shown by streptomycin in infections produced by certain pathogenic bacteria, and also in the lower toxicity of streptomycin.

The present report is mainly concerned with the comparative value of streptomycin and streptothricin as chemo-therapeutic agents in the treatment of gram-negative and gram-positive infections in mice.

Materials and Methods. With the exception of the streptomycin, the materials and methods of the *in vitro* and *in vivo* assays have been reported in previous communications, and will therefore not be described in detail here.[6] The streptothricin W and streptothricin F used were the same as those reported in a recent paper.[11] The streptomycin was kindly supplied by Dr. S. A. Waksman of Rutgers University, and Drs. M. Tishler and R. Denkewalter of the Research Laboratories of Merck & Co., Inc. The potency of this substance was 27 to 100 units per mg of solid, one unit being that quantity of streptomycin which will just inhibit a given strain of *E. coli* in 1 ml of nutrient broth or agar, as described by Waksman *et al.*[10] The streptothricin unit differs somewhat from the streptomycin unit and has been described by Foster *et al.*[3] The inhibitory action of these substances on the growth of bacteria and fungi *in vitro* was determined by incorporating the drugs in agar and streaking the surface of the

1 Waksman, S. A., and Woodruff, H. B., *J. Bact.*, 1940, **40**, 581.

2 Waksman, S. A., and Woodruff, H. B., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 609.

3 Foster, J. W., and Woodruff, H. B., *Arch. Biochem.*, 1943, **3**, 241.

4 Robinson, H. J., Thesis, Rutgers University, 1943.

5 Robinson, H. J., Graessle, O. E., and Smith, D. G., *Science*, 1944, **99**, 540.

6 Robinson, H. J., and Smith, D. G., *J. Pharm. and Exp. Therap.*, in press.

7 Foster, J. W., and Woodruff, H. B., in press.

8 Schatz, A., and Waksman, S. A., in press.

9 Schatz, A., Bugie, E., and Waksman, S. A., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 66.

10 Jones, D., Metzger, H. J., Schatz, A., and Waksman, S. A., *Science*, 1944, **100**, 103.

11 Robinson, H. J., Graessle, O. E., and Silber, R. H., *J. Pharm. and Exp. Therap.*, in press.

TABLE I.
Acute Subcutaneous Toxicity for Mice, Streptomycin and Streptothricin.

Drug	No. of mice	Dose in units per 20 g	No. dead Time in days 1	2	3	4	5	6	7	8	9	10	% dead
Streptothricin	15	1250	0	0	0	0	0	0	0	0	0	0	0
"	15	2500	0	0	0	0	2	0	2	0	2	0	40
"	15	5000	0	0	2	2	4	3	2	2	—	—	100
Streptomycin (low potency)	20	1250	0	0	0	0	0	0	0	0	0	0	0
" " "	25	2500	6	1	0	0	0	0	0	0	0	0	28
" " "	20	5000	16	2	0	0	0	0	0	0	0	0	90
Streptomycin (high potency)	15	5000	0	0	0	0	0	0	0	0	0	0	0
" " "	15	7500	0	0	0	0	0	0	0	0	0	0	0
" " "	15	10,000	0	0	0	0	0	0	0	0	0	0	0
" " "	10	15,000	9	0	0	0	0	0	0	0	0	0	90

agar with the test organism. The rate at which streptomycin killed bacteria was determined in defibrinated rabbit blood or broth, using the rotating rack technic as described in earlier papers.[12]

Acute Toxicity. A summary of the acute toxicity tests performed with streptomycin and streptothricin is presented in Table I. The results show that certain batches of streptomycin are considerably less toxic than streptothricin. A further difference between the two substances appeared to be that death following the injection of streptomycin occurs within 48 hours after the injection, whereas in the case of streptothricin W the majority of deaths occur after the third day. The toxic signs manifested by streptothricin have been reported elsewhere, and therefore will not be described here.[11] The visible responses of mice to toxic doses of streptomycin consisted of increased activity, marked dypsnea, and in some animals death due to respiratory failure. It was observed that the more toxic preparations of streptomycin lowered the blood pressure of rabbits in the same manner as streptothricin.[11] On the other hand, the least toxic lot of streptomycin had no effect upon the blood pressure of rabbits, even in doses as large as 12,000 units per kg. These findings suggest that under certain conditions streptomycin may be contaminated with a toxic factor which is lethal for mice, and causes a precipitous fall in blood pressure of rabbits.

Results in vitro—*Agar Plate.* The results of the bacteriostatic tests are summarized in Table II and show that the spectrum of activity of streptomycin is in many respects identical with that of streptothricin. Both substances show remarkable activity against both gram-positive and gram-negative pathogens.

TABLE II.
Bacteriostatic Action of Streptomycin and Streptothricin.
Agar Plate Method.

Organism	Factors for inhibition Streptomycin	Streptothricin
**E. coli* "W"	1	1
E. coli "C"	1	0.5
Strep. hemolyticus MIT	> 16	> 16
Strep. hemolyticus M	2	16
Strep. viridans	> 16	> 16
Strep. lactis	4	4
Staph. aureus SM	0.5	4
Staph. aureus FDA	1	1
Staph. aureus SD	> 16	1
Staph. aureus 155	2	4
Diplo. pneumoniæ II M	8	4
B. mycoides	0.5	> 16
B. subtilis	0.25	0.5
E. typhi	1	0.25
S. aertrycke	4	2
S. enteritidis	0.5	0.5
S. schottmülleri	2	2
B. flexneri II	0.25	1
B. sonne	1	1
P. leptisepticum	0.5	0.5
B. proteus S	4	16
B. proteus	4	8
B. pyocyaneus II	> 16	> 16
B. pyocyaneus III	> 16	> 16
A. aerogenes	0.5	1
Cl. welchii WX	>104	>104
Cl. tetani	>104	>104
Cl. septicum	>105	52
Cl. sordelli	>105	>104

* 7.5 units per 1 cc of agar required to inhibit *E. coli* "W."

[12] Robinson, H. J., *J. Pharm. and Exp. Therap.*, 1943, **77**, 70.

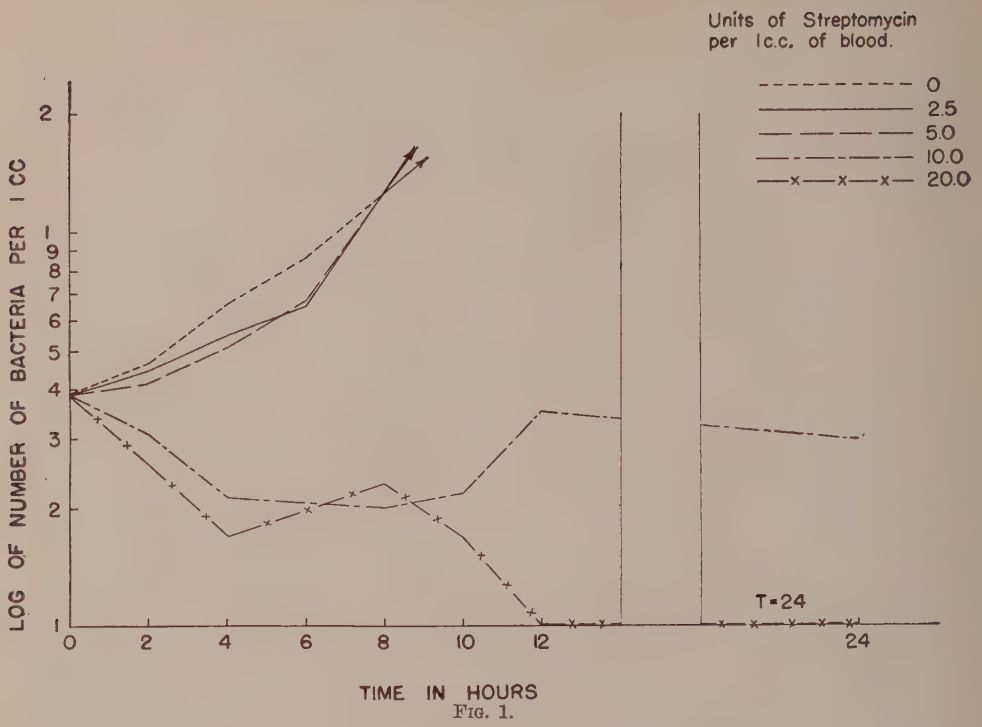

FIG. 1.

However, a notable difference between streptomycin and streptothricin was noted in their respective action against certain bacterial strains, even within the same species. Thus, streptomycin was 8 times more effective than streptothricin against *Staph. aureus SM,* but 16 times less effective against *Staph. aureus SD, B. mycoides, B. flexneri, B. proteus, A. aerogenes,* and certain strains of staphylococci and streptococci appeared to be more sensitive to streptomycin than to streptothricin. Neither of the substances was very active against the gram-positive anaerobes.

Pathogenic and saprophytic fungi were much more susceptible to the action of streptothricin (Table III). In concentrations of 250 units per cc of agar the latter was fungistatic for most strains, whereas the same concentration of streptomycin appeared to be without effect. When higher concentrations of streptomycin were employed, however, streptomycin also showed fungicidal properties.

TABLE III.

Bacteriostatic Action of Streptomycin and Streptothricin Against Fungi.
Agar Plate Method—Sabouraud's Agar.

Organism	No. of units per cc agar required to cause inhibition	
	Streptomycin	Streptothricin
Aspergillus niger MF II	>4,000	250
Penicillium chrysogenum MF 56	>4,000	250
Cryptococcus neoformans No. 3709	>4,000	250
Epidermophyton inguinale	>4,000	1,000
Microsporum canis No. 232	4,000	1,000
Sporotrichum schenki No. 7017	>4,000	250
Trichophyton gypseum	3,500	500
Trichophyton interdigitale No. 640	4,000	1,000

Cultures were incubated at 29°C for 14 days.

Rotating Rack Technic. When the test organism was cultured in blood or broth in the presence of streptomycin or streptothricin,

TABLE IV.

Efficacy of Streptomycin in Mice Infected with *S. schottmülleri*. Comparison of Intravenous, Intraperitoneal, Subcutaneous, and Oral Therapy.

Organism: *Salmonella schottmülleri.*
Age of Culture: 6 hours.
Infection: 0.5 cc of a 10^{-5} dilution in 4% mucin.
Therapy: Streptomycin given intravenously, intraperitoneally, subcutaneously, or orally immediately after inoculation.

No. of mice	Drug	Units per dose	No. doses per day	Culture dilution	No. surviving in days 1	2	3	4	5	6	7	8	9	10	% survival
				Therapy: A single intravenous dose.											
10	Streptomycin	3.125	1	10^{-5}	1	0	0	0	0	0	0	0	0	0	0
10		6.25	1	"	2	1	1	0	0	0	0	0	0	0	0
10		12.5	1	"	3	3	1	1	1	1	1	1	1	1	10
10		25.0	1	"	5	2	2	2	2	1	1	1	1	1	10
10		50.0	1	"	9	7	6	6	6	6	6	6	6	6	60
10		100.0	1	"	10	10	10	10	10	9	9	9	9	9	90
10		200.0	1	"	10	10	10	10	10	10	10	10	10	10	100
				Therapy: A single intraperitoneal dose.											
30	Streptomycin	3.125	1	10^{-5}	5	4	3	3	2	2	2	2	2	2	6.7
30		6.25	1	"	13	6	4	2	2	2	2	2	2	2	6.7
30		12.5	1	"	25	21	20	19	18	18	17	17	16	16	52.8
30		25.0	1	"	28	26	26	26	26	26	24	24	23	23	75.9
30		50.0	1	"	29	29	29	29	29	29	29	29	29	29	96.7
				Therapy: A single subcutaneous dose.											
30	Streptomycin	6.25	1	10^{-5}	3	3	3	3	3	3	3	3	3	3	9.9
30		12.5	1	"	7	6	5	4	3	3	2	2	2	2	6.6
30		25.0	1	"	16	13	10	9	6	6	6	6	6	6	19.8
30		50.0	1	"	28	28	28	28	27	27	26	26	26	26	85.8
30		100.0	1	"	30	30	29	29	29	29	29	29	29	29	96.7
				Therapy: A single oral dose.											
10	Streptomycin	93.75	1	10^{-5}	4	2	2	2	0	0	0	0	0	0	0
10		187.5	1	"	1	1	1	0	0	0	0	0	0	0	0
10		375.0	1	"	2	0	0	0	0	0	0	0	0	0	0
10		750.0	1	"	3	2	2	2	1	1	1	1	1	1	10
10		1500.0	1	"	9	7	7	7	6	6	6	6	6	6	60
10		3000.0	1	"	10	10	9	9	8	8	8	8	8	8	80
				Therapy: None.											
40	Controls	—	—	10^{-5}	0	0	0	0	0	0	0	0	0	0	0
20		—	—	10^{-6}	6	2	2	1	1	1	1	1	0	0	0
20		—	—	10^{-7}	12	7	7	7	6	5	4	3	3	3	15
20		—	—	10^{-8}	15	8	7	5	5	5	5	4	4	4	20

and the rate of killing estimated by counting the surviving organisms at frequent intervals, it was found that concentrations of 20 units of either drug per cubic centimeter of blood produced a gradual killing effect upon *S. schottmülleri* which sterilized the culture within 12 hours (Fig. 1). Concentrations of 10 units per cc exerted a bacteriostatic effect, whereas lower concentrations had little inhibitory effect. Similar findings were obtained with other organisms, including *E. coli, S. aertrycke,* and *E. typhi.*

Microscopic examination of the cultures containing streptomycin or streptothricin revealed an elongation of the majority of the cells such as that reported following exposure to penicillin,[13], streptothricin,[3] and the sulfonamides.[14] The morphological changes were induced at dilutions far greater than the minimal inhibitory dilution, and suggest that very small amounts of streptomycin and streptothricin may influence the normal rate of division and hence the pathogenicity of the organism.

Results in vivo. Since the *in vivo* findings with a number of bacterial strains of the colon-typhoid and salmonella group were essentially the same, only the results with one bacterial strain are presented in Table IV. Single doses of 50 to 100 units of streptomycin

[13] Smith, L. D., and Hay, T., *J. Franklin Inst.*, 1942, **233**, 598.

[14] Lockwood, J. S., *J. Immun.*, 1938, **35**, 155.

TABLE V.
Efficacy of Streptomycin in Mice Infected with *S. schottmülleri*. Subcutaneous Therapy.

Organism: *Salmonella schottmülleri.*
Age of Culture: 6 hours.
Infection: 0.5 cc of a 10^{-5} dilution in 4% mucin.
Therapy: Streptomycin or streptothricin given subcutaneously immediately after bacterial inoculation.

No. of mice	Drug	Units per dose	No. doses per day	Culture dilution	No. surviving in days 1	2	3	4	5	6	7	8	Survival %
				Therapy: A single dose.									
10	Streptomycin	12.5	1	10^{-5}	2	2	2	2	2	1	1	1	10
10		25.0	1	"	8	8	8	7	7	7	7	7	70
10		50.0	1	"	10	10	10	9	9	9	9	9	90
10		100.0	1	"	9	9	9	9	9	9	9	9	90
10	Streptothricin	12.5	1	10^{-5}	4	3	2	2	2	2	2	2	20
10		25.0	1	"	5	4	4	4	4	4	4	4	40
10		50.0	1	"	10	10	10	9	9	9	9	9	90
10		100.0	1	"	10	10	10	10	10	10	10	10	100
				Therapy: Every 6 hr over a 24-hr period.									
20	Streptomycin	12.5	4	10^{-5}	15	12	12	12	12	12	11	11	55
20		25.0	4	"	20	19	18	18	18	18	18	18	90
20		50.0	4	"	20	20	20	20	20	20	20	20	100
10	Streptothricin	12.5	4	10^{-5}	9	5	4	4	4	3	3	3	30
10		25.0	4	"	10	10	10	10	9	9	9	9	90
10		50.0	4	"	10	10	10	10	10	10	9	9	90
10		100.0	4	"	10	10	10	10	10	10	10	10	100
				Therapy: None.									
20	Controls	—	—	10^{-5}	0	0	0	0	0	0	0	0	0
10		—	—	10^{-6}	6	4	3	3	3	3	3	3	20
15		—	—	10^{-7}	10	8	7	6	6	6	6	5	33
15		—	—	10^{-8}	12	10	10	10	10	9	9	9	60

per mouse given intravenously or subcutaneously immediately after the bacterial inoculation, were sufficient to protect a large percentage of the mice from a lethal infection of *S. schottmülleri.* When given by intraperitoneal injection, the drug was even more efficacious than following subcutaneous or intravenous administration. As reported for streptothricin,[5] streptomycin was found to be much less effective by mouth than following parenteral administration.

Multiple doses of streptomycin given every 6 hours over a 24-hour period appeared to afford essentially the same protection as equal quantities of the drug given as a single dose immediately after the bacterial inoculation. Essentially the same results were obtained with streptothricin (Table V).

Infections produced by certain gram-positive bacteria were found to be somewhat more resistant to streptomycin than were the infections caused by the foregoing gram-negative bacteria. Nevertheless, infections caused by *Diplococcus pneumoniæ* and *Staphylococcus aureus* were readily controlled by adequate doses of streptomycin (Table VI.) Under the same conditions, streptothricin was not efficacious.

Discussion. Streptomycin, like streptothricin, would seem to offer promise as a chemotherapeutic agent for the treatment of a variety of bacterial infections. Streptomycin seems to possess some advantages over streptothricin, particularly from the point of view of toxicity and its action *in vivo* against gram-positive bacteria. Although some batches of streptomycin were as toxic as streptothricin, the findings suggest that under the proper conditions a relatively non-toxic preparation can be obtained. The toxic signs manifested by mice injected with lethal doses of streptomycin were in many respects similar to those produced by streptothricin F, a preparation recently described by Robinson, Graessle and Silber[11] which was obtained from a medium containing corn steep liquor. The nature of the toxic signs produced by streptomycin and streptothricin F appears to be identical and

TABLE VI.

Efficacy of Streptomycin in Mice Infected with *D. pneumoniæ*. (Subcutaneous Therapy.)

Organism: *Diplococcus pneumoniæ* Type I, No. 37.
Age of Culture: 6 hours.
Infection: 0.5 cc of a 10^{-6} culture dilution in broth.
Therapy: Streptomycin or streptothricin given subcutaneously immediately after bacterial inoculation.

No. of mice	Drug	Units per dose	No. doses per day	Culture dilution	No. surviving in days 1	2	3	4	5	6	7	8	Survival %
				Therapy: A single dose.									
10	Streptomycin	50	1	10^{-6}	1	0	0	0	0	0	0	0	0
10		100	1	"	1	0	0	0	0	0	0	0	0
20		200	1	"	1	0	0	0	0	0	0	0	0
20		400	1	"	9	2	2	1	1	1	1	1	5
20		800	1	"	19	6	6	5	5	5	5	5	25
20		1600	1	"	20	20	20	20	20	20	20	20	100
10		3200	1	"	10	10	10	10	10	10	10	10	100
10		6400	1	"	10	10	10	10	10	10	10	10	100
				Therapy: A single dose.									
20	Streptothricin	200	1	10^{-6}	0	0	0	0	0	0	0	0	0
20		400	1	"	3	0	0	0	0	0	0	0	0
20		800	1	"	14	2	2	0	0	0	0	0	0
20		1600	1	"	19	11	8	5	3	3	2	2	10
				Therapy: None.									
30	Controls	—	—	10^{-6}	0	0	0	0	0	0	0	0	0
20		—	—	10^{-7}	2	0	0	0	0	0	0	0	0
20		—	—	10^{-8}	4	0	0	0	0	0	0	0	0
20		—	—	10^{-9}	6	0	0	0	0	0	0	0	0

seems to be due to a histamine-like substance present in the toxic preparations. To date, sufficient quantities of streptomycin have not been available for extensive toxicological and pharmacological studies. Until such studies are completed, the value of this substance for the treatment of bacterial disease in animals and man cannot be fully evaluated. Although streptomycin appears to have a number of advantages over streptothricin, the latter is much more effective against fungi.

Summary. Streptomycin seems to possess some advantages over streptothricin particularly from the point of view of toxicity and its greater action against certain gram-negative and gram-positive bacteria *in vivo*. Although certain batches of streptomycin appear to have the same order of toxicity as streptothricin, the findings suggest that under proper conditions a relatively non-toxic preparation can be obtained. Streptothricin seems to be much more effective *in vitro* against pathogenic fungi than streptomycin.

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Effect of Feeding Dried Milk on Production of Liver Cancer by *p*-Dimethylaminoazobenzene.

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Dietary factors greatly influence the formation of liver cancer by *o*-aminoazobenzene or *p*-dimethylaminoazobenzene (butter yellow). Experiments have shown that the incidence of liver cancer in rats caused by these chemicals is definitely reduced when the basal diet consists of wheat,[1] rye,[2] or millet seed[3] instead of rice.

[1] Ando, T., *Gann*, 1938, **32**, 252.

[2] Maisin, J., *Bull. Assoc. franc. p. l'étude du cancer*, 1940, **29**, 6.

TABLE I.
Incidence of Hepatic Tumors in Rats Fed Different Amounts of Dried Whole Milk.

Diet	No. of animals used	No. of days fed	Liver findings*					Incidence of liver cancer, %
			—	±	+	++	+++	
Butter yellow and rice	39	100-200	0	0	5	23	11	100
Butter yellow and rice and 6% Klim	20	150-163	2	0	0	14	4	90
Butter yellow and rice and 15% Klim	20	154-198	5	3	4	4	4	60
Butter yellow and rice and 20% Klim	20	132-227	10	0	2	4	4	50

* — indicates smooth, practically normal liver; ± indicates nodular cirrhosis with adenomatous hyperplasia; + indicates distinct areas of cholangioma or hepatoma, or both; ++ indicate extensive liver cancer without metastases; +++ indicate extensive liver cancer with metastases.

It is also known that the addition of yeast,[4,5,6] or liver[7] to a rice diet increases resistance to this type of tumor formation.

It has also been reported that *p*-dimethylaminoazobenzene will fail to produce liver cancer in rats maintained on a rice diet supplemented with either casein and riboflavin,[8] casein and B vitamins,[9] or cystine and choline.[10]

The experiments described in this report were undertaken to determine the effect of dried milk feeding on the incidence of hepatic tumors in rats after feeding *p*-dimethylaminoazobenzene.

Experimental. In the present study, dried whole milk* was chosen because of its high casein and riboflavin content (28.0% fat, 26.7% milk protein and 1.6 mg riboflavin per 100 g).†

Three groups of 20 rats each (125 to 150 g body weight) were maintained on a basal diet of unpolished rice mixed with 6, 15, and 20% of dried whole milk, respectively. A control group of 50 rats was kept on unpolished rice alone. All animals received 3% *p*-dimethylaminoazobenzene in cottonseed oil in the proportion of 20 cc of the solution per kg of ration noted above. All of the 4 groups of rats received a small amount of fresh carrots daily. Unlimited water was allowed. Feeding on the various diets was continued for about 200 days; all rats then living were sacrificed and examined.

The results obtained from this study are summarized in Table I. Rats that died before the period of 100 days are not included in the table. In all cases the gross diagnosis was confirmed by microscopic examination. Rats used in this study were of the Sherman stock.

As may be seen in Table I, the addition of 15 and 20% of dried whole milk to unpolished rice had a distinct protective effect, though not absolute, against the pathological changes leading to the development of liver cancer. Among 20 rats fed 20% dried whole milk, 10, or 50%, had grossly and histologically normal livers; the remaining 10 rats showed numerous large and small tumor nodules with or without metastases in the mesentery and omentum. On the other hand, the animals fed the azo dye-rice diet without Klim developed typical liver cancer in all cases in comparatively shorter periods of time. The addition of 6% Klim to the butter yellow-rice diet, however, had practically no inhibitory effect upon the production of liver cancer.

Animals on the butter yellow-rice diet containing 20% dried whole milk consumed from 7 to 9 g of the food daily. Therefore, a rat

[3] Morigami, S., and Kasiwabara, N., *Gann*, 1941, **35**, 65.

[4] Kinosita, R., *J. Jap. Soc. Dis. Digest. Org.*, 1938, **37**, 513.

[5] Nakahara, W., Fujiwara, T., and Mori, K., *Gann*, 1939, **33**, 57.

[6] Sugiura, K., and Rhoads, C. P., *Cancer Research*, 1941, **1**, 3.

[7] Nakahara, W., Mori, K., and Fujiwara, T., *Gann*, 1938, **32**, 465; 1939, **33**, 406.

[8] Kensler, C. J., Sugiura, K., Young, N. F., Halter, C. R., and Rhoads, C. P., *Science*, 1941, **93**, 308.

[9] Miller, J. A., Miner, D. L., Rusch, H. P., and Baumann, C. A., *Cancer Research*, 1941, **1**, 699.

[10] György, P., Poling, E. C., and Goldblatt, H., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 41.

* Klim, manufactured by The Borden Co., New York.

† The author is indebted to Dr. Raymond Hertwig of The Borden Co. for information regarding the composition of the powdered whole milk used.

consumed approximately 0.4 g of casein and 26 μg of riboflavin daily.

The inhibitory effect of dried whole milk feeding upon the production of liver cancers is distinctly less than that of the combination of purified casein and crystalline synthetic riboflavin, or the feeding of a diet of unpolished rice containing 15% of brewers' yeast (50% against 93 and 100% protection, respectively, at 200 days). Part of the difference in the protective effect in these cases may be due to variations in the vitamin levels (daily ingestion of 26 γ against 200 and 84 γ riboflavin, respectively).

In order to eliminate the possible tumor-promoting activity of the high-fat content of Klim the following experiment was undertaken. Forty young adult rats were maintained on the butter yellow diet of 85% rice and 15% of dried skimmed milk (Powdered Skimmed Milk, The Borden Co., New York.) It contained 1.0% fat, 36.5% protein, and 1.9 mg riboflavin per 100 g.

The results showed that the reduction in the amount of fat content in the diet did not increase the protective action of dried milk against liver cancer formation. Of the 34 rats which died or were sacrificed between 150 and 250 days after the beginning of the experiment, 14, or 41%, had grossly and microscopically normal livers; and 20, or 59%, had liver cancer, both cholangioma and hepatoma.

The majority of the animals fed the butter yellow-rice diet with dried whole milk gained weight and presented a healthy appearance throughout the experimental period.

Effect of Adding Dried Whole Milk to the Basal Rice Diet Following Periods of Feeding a Butter Yellow-Rice Diet. A recent study[11] showed that a majority of rats fed a butter yellow-rice diet for 60 days developed cirrhosis of the liver. At this time, 20% of the livers also showed adenomatous hyperplasia of the bile ducts with beginning malignant transformation into cholangiomas, or early hepatoma formation. The withdrawal of butter yellow from the diet at this stage did not prevent the subsequent development of liver cancers. However, this precancerous condition (cirrhosis of the liver) was abolished to a large extent by a rice diet containing 15% yeast.[11]

The succeeding paragraphs deal with attempts to ascertain whether a destructive action against experimental liver tumors can be accomplished by the addition of dried whole milk to the basal diet after the formation of hepatic cancers, or precancerous states, resulting from the previous ingestion of *p*-dimethylaminoazobenzene.

Following the procedure elsewhere described,[11] the author maintained rats on a butter yellow-rice diet for definite periods of time. Then the carcinogen was removed from the food and feeding was continued on the basal unpolished rice containing a supplement of 15% dried whole milk, until the animals either succumbed or were sacrificed.

The results are presented in Table II.

The data in Table II show clearly that dried whole milk feeding produced a striking inhibition of liver cancer development. In 20 rats which received the preliminary feeding with butter yellow for 62 days, followed by rice-dried whole milk diet without butter yellow for 113 to 168 days, 18, or 90%, had smooth, practically normal livers. In contrast, of animals fed the butter yellow-rice diet for the same period, followed by rice alone, only 12% had histologically normal livers.

If the preliminary feeding with butter yellow exceeded 88 days, liver cancer resulted in almost 100% of the cases. These tumors also showed metastases in the mesentery and omentum, even though the dietary anticarcinogen (powdered whole milk) was fed for long periods of time subsequent to butter yellow ingestion.

Aspiration biopsies were performed on the livers in 34 cases to establish the presence of liver cirrhosis and tumors. About 60% of the livers of rats maintained on butter yellow-rice diet for 60-70 days gave evidence of cirrhosis, while about 75% of the livers of rats maintained on butter yellow-rice diet for 100 days or longer gave positive evidence of tumor formation.

Summary. 1. The therapeutic action of dried whole milk upon liver cirrhosis and cancer has been investigated. The liver changes were

[11] Sugiura, K., and Rhoads, C. P., *Cancer Research*, 1942, **2**, 453.

TABLE II.
Results of Adding Dried Whole Milk Following Periods of Butter Yellow-Rice Diet, on the Production of Liver Cancer.

No. of animals used	No. of days fed on butter yellow and rice	No. of days fed rice and Klim diet subsequent to butter yellow-rice diet	Liver changes*				
			—	±	+	++	+++
20	62	113-168	18	0	0	0	2
27	88	100-250	1	0	0	5	21
14	124	82-254	0	0	2	6	6

* See Table I for explanation of symbols.

induced in rats by feeding unpolished rice and *p*-dimethylaminoazobenzene. 2. The production of liver cancer in rats fed *p*-dimethylaminoazobenzene was partially inhibited by the daily ingestion of dried whole milk (50% protection at 200 days). 3. Liver cirrhosis produced by *p*-dimethylaminoazobenzene has been treated successfully by a rice diet containing 15% dried whole milk. (Based on previous evidence of the high incidence of liver cirrhosis in rats produced by *p*-dimethylaminoazobenzene-rice diet.[11]). 4. Once adenomatous hyperplasia of bile ducts, cholangioma, or hepatoma had been established in the livers, these benign and malignant tumors could not be destroyed by ingestion of the rice-milk diet.

The author wishes to express his appreciation to Dr. C. P. Rhoads for his interest and valuable advice.

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Effect of Malonate, Fumarate, Succinate, and Citrate on Synthesis of Acetylcholine *in vitro*.*

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The increased synthesis of acetylcholine *in vitro* under aerobic conditions is greater in the presence of serum and spinal fluid obtained from healthy humans than from patients with myasthenia gravis.[1,2,3] Since the synthesis of acetylcholine *in vitro* is increased in the presence of glucose and pyruvate[4] the following experiments were designed to ascertain whether or not possible intermediates of glucose and pyruvate metabolism modify the synthesis of acetylcholine.

Method. The synthesis of acetylcholine was studied by the method of Quastel, Tennenbaum, and Wheatley[4] with minor modifications.[2] Varying amounts of sodium succinate, sodium fumarate, sodium malonate, sodium citrate, and sodium oxalate were added to mixtures containing 100 mg minced fresh frog brain, 1 cc serum or spinal fluid, 3 mg physostigmine salicylate, and 2 cc Ringer's solution. The pH of the mixtures was adjusted to 7.4. Identical mixtures without the salts of these organic acids served as controls. The mixtures were shaken and incubated aerobically for 4 hours at 37°C. After incubation the amounts of free and total acetylcholine synthe-

* This study was aided by a grant from the Josiah Macy, Jr., Foundation.

1 Torda, C., and Wolff, H. G., *Science*, 1943, **98**, 224.

2 Torda, C., and Wolff, H. G., *J. Clin. Invest.*, 1944, **23**, 649.

3 Torda, C., and Wolff, H. G., *Science*, 1944, **100**, 200.

4 Quastel, J. H., Tennenbaum, M., and Wheatley, A. H. M., *Bioch. J.*, 1936, **30**, 1668.

TABLE I.
Effect of the Substances on the Synthesis of Acetylcholine *in vitro*.

Substance	Final concentrations in mols	Amount of acetylcholine synthesized in % of control*			
		Free acetylcholine In the presence of frog brain and		Total acetylcholine In the presence of frog brain and	
		Spinal fluid	Serum	Spinal fluid	Serum
Sodium malonate	8×10^{-5}	66	70	64	62
	8×10^{-4}	55	54	54	61
	8×10^{-3}	53	50	51	58
" fumarate	8×10^{-5}	131	134	125	126
	8×10^{-4}	138	140	130	130
	8×10^{-3}	142	151	140	147
" succinate	5×10^{-5}	143	136	140	136
	5×10^{-4}	151	150	155	149
	5×10^{-3}	145	142	148	143
" citrate	3.5×10^{-5}	130	135	129	128
	3.5×10^{-4}	140	142	139	138
	3.5×10^{-3}	150	154	150	147
" oxalate	4×10^{-5}	92	88	102	99
	4×10^{-4}	105	110	95	98
	4×10^{-3}	100	103	107	109

* Each value represents the average of 10 separate experiments. The S.E. of the mean for each value was less than ±8%. The amounts of acetylcholine synthesized in μg per 100 mg frog brain, followed by the S.E. of the mean were: spinal fluid: free, 1.57 ± 0.048; total, 2.36 ± 0.052; serum: free, 1.15 ± 0.045; total, 1.69 ± 0.054.

sized were assayed biologically on the sensitized rectus abdominis muscle of the frog. Since the compounds in the concentrations used did not modify the effect of acetylcholine in inducing muscle contraction, this procedure should be a valid indicator of the acetylcholine content of the mixtures.

Results. The amounts of acetylcholine synthesized in the presence of the substances used are given in Table I. Sodium malonate decreased the amount of acetylcholine synthesized, while succinate, fumarate, and citrate increased the synthesis of acetylcholine.

To determine whether or not the increased synthesis with sodium citrate was due to the removal of calcium ion, sodium oxalate was added in concentrations large enough to remove more calcium than did citrate, but still small enough to be bound mainly by the calcium present in the mixtures and therefore without any effect of its own upon the synthesis of acetylcholine. Since the synthesis of acetylcholine was not significantly modified in the presence of sodium oxalate in the concentrations used, the effect of sodium citrate was not due to decreased calcium ion concentration in the incubated mixtures.

Discussion. In the presence of the above intermediates of glucose and pyruvate metabolism in small concentrations the synthesis of acetylcholine was increased by about 50%. Succinate in larger concentrations and under different oxygen tension may have a different effect.[4,5,6] Malonate, known to depress the oxidation of succinate,[7,8] also depresses the synthesis of acetylcholine.

For the mechanism of action of fumarate, succinate, and citrate it is possible that these substances in small concentrations increase the synthesis of acetylcholine because they are

5 Nachmansohn, D., John, H. M., and Waelsch, H., *J. Biol. Chem.*, 1943, **150**, 485.

6 Furchgott, R., and Schorr, E., personal communications.

7 Cook, R. P., *Bioch. J.*, 1930, **24**, 1538.

8 Quastel, J. H., and Wheatley, A. H. M., *Bioch. J.*, 1931, **25**, 117.

converted to some compounds[9] that can be utilized for the acetylation of choline or because of energy liberated during the metabolism of fumarate, citrate, and succinate.

[9] Evans, E. A., Jr., *Harvey Lectures*, 1944, **39**, 273.

Summary. 1. The synthesis of acetylcholine *in vitro* in the presence of sodium malonate, succinate, citrate, and fumarate was investigated. 2. Malonate decreased the synthesis of acetylcholine in the concentrations used, while citrate, succinate, and fumarate increased it.

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Effect of Vitamin K (Menadione) on Choline Esterase Activity, Acetylcholine Synthesis, and Striated Muscle.*

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Under physiological conditions menadione (2-methyl-1,4-naphthoquinone) has the ability to react with substances containing the –SH group.[1,2,3] Therefore the activity of some enzymes having an active –SH group may be modified by menadione. Since choline esterase[4] and the enzyme that synthesizes acetylcholine[5] probably contain an active –SH group, a study was made to ascertain whether or not menadione modifies the activity of these enzymes.[6]

Experimental. I. *Effect of menadione on the Synthesis of Acetylcholine.* The synthesis of acetylcholine was studied by the method of Quastel, Tennenbaum, and Wheatley[7] with minor modifications.[8] Varying amounts of menadione were added to mixtures containing 100 mg minced fresh frog brain, 3 cc Ringer's solution, 3 mg physostigmine salicylate and 4.8 mg glucose. The pH of the mixtures was adjusted to 7.4. Identical mixtures without menadione served as controls. The mixtures were shaken and incubated aerobically for 4 hours. After incubation the amounts of free and total acetylcholine synthesized were assayed biologically on the sensitized rectus abdominis muscle of the frog.

Menadione depressed the synthesis of acetylcholine and the decrease was more marked in the presence of larger concentrations of the vitamin (see Table I).

In another series of experiments the amount of substrate was increased by adding 1 cc of spinal fluid to the mixtures before incubation. In such mixtures more acetylcholine is synthesized.[9] In the presence of menadione the synthesis of acetylcholine was decreased; the percentage decrease being similar to that found in the absence of spinal fluid (Table I).

Methylene blue and menadione have similar effects on oxidative processes. Therefore the effect of methylene blue on the synthesis of acetylcholine was investigated. In the presence of 1×10^{-6} mol. methylene blue the synthesis of acetylcholine was 20% less (average of 6 series of experiments). Since methylene blue greatly increased the sensitivity of the

* This study was aided by a grant from the Josiah Macy, Jr., Foundation.

[1] Voegtlin, C., Rosenthal, S. M., and Johnson, J. M., *U. S. Public Health Reports*, 1931, **46**, 339.

[2] Fieser, L. F., *Ann. Int. Med.*, 1941, **15**, 648.

[3] Summerson, W. H., *Fed. Proc.*, 1943, **2**, 72.

[4] Nachmansohn, D., and Lederer, E., *Bull. Soc. Chem. Biol.*, 1939, **21**, 797.

[5] Nachmansohn, D., and Machado, A. L., *J. Neurophysiol.*, 1943, **6**, 397.

[6] Torda, C., and Wolff, H. G., *Fed. Proc.*, 1944, **3**, 86.

[7] Quastel, J. H., Tennenbaum, M., and Wheatley, A. H. M., *Bioch. J.*, 1936, **30**, 1668.

[8] Torda, C., and Wolff, H. G., *J. Clin. Invest.*, 1944, **23**, 649.

[9] Torda, C., and Wolff, H. G., *Science*, 1944, **100**, 200.

TABLE I.
Effect of Menadione on the Synthesis of Acetylcholine *in vitro*.

Concentration of menadione (mols)	Amounts of acetylcholine synthesized in μg per 100 mg frog brain			
	Free acetylcholine In the presence of frog brain and		Total acetylcholine In the presence of frog brain and	
	Ringer's solution*	Spinal fluid†	Ringer's solution*	Spinal fluid†
0	0.070 ± 0.023	1.57 ± 0.048	1.50 ± 0.044	2.36 ± 0.052
	Amounts of acetylcholine synthesized in percent of these controls.‡			
0	100	100	100	100
2×10^{-6}	86	83	83	84
2×10^{-5}	67	76	75	76
2×10^{-4}	61	61	70	63
2×10^{-3}	30	23	26	20

* Mixtures incubated at 23°C.
† Mixtures incubated at 37°C.
‡ Each value represents the average of 5 separate experiments. The S.E. of the mean for each value was less than ±5%.

eserinized rectus abdominis muscle to acetylcholine, the amounts of acetylcholine synthesized in the presence of larger concentrations of methylene blue are difficult to evaluate.

II. *Effect of Menadione on the Activity of Choline Esterase* (manometric method). Human serum or ox brains were used as a source of choline esterase. The brains were frozen and finely ground.[9] The tissue was pressed through muslin and suspended in bicarbonated Ringer's solution at pH 7.4. Two cc of the suspension of brain and menadione (from 0 to 0.774 mg) were placed in the body of the vessel of the Warburg apparatus and 0.2 cc of a 5% solution of acetylcholine in Ringer's solution was placed in the side cup. In another series of experiments instead of the suspension of brain 0.3 cc serum and 1.7 cc Ringer's solution were used. Mixtures without menadione and mixtures without menadione but containing physostigmine salicylate in various concentrations served as controls. The activity of the choline esterase was then ascertained manometrically by measuring the amount of CO_2 liberated during the hydrolysis of acetylcholine following the method of Ammon.[11]

Menadione did not modify the activity of choline esterase in concentrations from $1x10^{-7}$ to $1x10^{-3}$ mol., but in concentrations of $2x10^{-3}$ mol. the activity of choline esterase was depressed by 70% regardless of the origin of the enzyme (serum or brain). Under similar conditions a $5x10^{-7}$ mol. physostigmine salicylate solution caused a 70% inhibition (average of 20 series of experiments).

In the following it was ascertained whether or not menadione modifies the effect of acetylcholine and other chemical stimuli, such as potassium, in inducing muscle contraction.

III. *Menadione and the Effect of Acetylcholine in Inducing Muscle Contraction.* The rectus abdominis muscle of frog was excised and suspended in a muscle chamber containing 10 cc Ringer's solution. The muscle was then immersed in a solution of acetylcholine (50 μg per 100 cc Ringer's solution) for 2 minutes and the amount of contraction of the muscle was registered by an isotonic lever on a kymograph. The muscle was then washed with Ringer's solution for 10 minutes. This procedure was repeated until 3 successive exposures to the solution of acetylcholine gave similar responses. After this stabilization of the muscle was obtained, the muscle was washed for 5 minutes and immersed for 5 minutes in a series of Ringer's solutions containing menadione in increasing concentrations ($1x10^{-7}$ to $2x10^{-3}$ mol.).

The results are given in Table II. Menadione in concentrations from $1x10^{-7}$ to $1x10^{-3}$ mol. did not modify the effect of acetylcholine on the muscle. In concentrations of $2x10^{-3}$ mol. the effect of acetylcholine was increased (average 60%).

Methylene blue, a potent inhibitor of cho-

[10] Bernheim, F., and Bernheim, M. L. C., *J. Pharm. Exp. Therap.*, 1936, **57**, 427.

[11] Ammon, R., *Pflüger's Arch.*, 1933-34, **233**, 486.

TABLE II.
Effect of Menadione and Methylene Blue on the Contraction of Striated Muscle Induced by Acetylcholine and Potassium.

Concentration of the drugs in mols	Magnitude of contraction in percent of control.* Contraction induced with Acetylcholine		Potassium	
	In muscles immersed in Menadione	Methylene blue	Menadione	Methylene blue
0	100	100	100	100
1×10^{-7}	111	121	122	119
1×10^{-6}	104	213	182	157
1×10^{-5}	102	381	202	381
1×10^{-4}	108		295	
1×10^{-3}	99		464	
2×10^{-3}	162		470	

* Each value represents the average of 10 separate experiments. The S.E. of the mean for each value was less than ±5%.

line esterase,[12,13] increased the effect of acetylcholine on the rectus abdominis muscle in concentrations from $1x10^{-7}$ mol. (Table II).

IV. *Menadione and the Effect of Potassium in Inducing Muscle Contraction.* The rectus abdominis muscle of frog was prepared and contracted as described above except that a 20mM solution of potassium chloride was used instead of acetylcholine to induce a contraction of the muscle. The height of contraction induced by potassium cannot be compared to that induced by acetylcholine since these substances cause different physicochemical changes in the muscle cells.[14,15] The results given in Table II show that menadione significantly increased the magnitude of contraction induced by potassium. It is difficult to reverse the effect of menadione. Menadione and methylene blue similarly modify the effect of potassium (Table II).

V. *Effect of High Concentrations of Menadione on Striated Muscle.* $2.5x10^{-3}$ mol. solution of menadione initiated a muscle contraction in 3 minutes without an addition of any other agent. This concentration approximates a saturated solution of this substance. A similar contraction was induced by a $2.5x10^{-3}$ mol. methylene blue solution.

Discussion. The effects of menadione may be related to its ability to react with substances containing the –SH group;[1,2,3] its ability to inhibit the aerobic glycolysis;[3,16,17,18] and to its positive oxidation-reduction potential.[16] This assumption is based on a comparison of the effect of menadione with that of methylene blue.

The above results suggest that menadione may depress the activity of organs stimulated by acetylcholine since the synthesis of acetylcholine is depressed by low concentrations of this vitamin. This effect is probably not counteracted by the depression of the activity of choline esterase since the concentrations of menadione required for the latter are much higher. The sensitivity of the effector cells to some chemical stimuli may be increased by menadione, for example, the effect of potassium on striated muscle is increased several times. It is likely that menadione cannot occur in the striated muscle of the living body in concentrations high enough to induce contractions.

Summary. 1. The effect of menadione in concentrations from $1x10^{-7}$ to $2.5x10^{-3}$ mol. on the synthesis of acetylcholine, on the activity of choline esterase, and on the effect of some chemical agents in inducing muscle

12 Rentz, E., *Arch. Exp. Path. Pharm.*, 1940, **196**, 148.

13 Bernheim, F., *The Interaction of Drugs and Cell Catalysts*, 1942, Burgess Publ. Co., Minneapolis, Minn.

14 Gasser, H. S., *Physiol. Rev.*, 1930, **10**, 35.

15 Henny, G. C., Ashkenaz, E. W., and Spiegel-Adolf, M., *Fed. Proc.*, 1944, **3**, 58.

16 Warren, C. O., *Am. J. Physiol.*, 1943, **139**, 719.

17 Fosdick, L. S., Hancher, O. E., and Calandra, J. C., *Science*, 1942, **96**, 45.

18 Armstrong, W. D., and Knutson, J. W., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 307.

contraction was investigated. 2. In the presence of menadione the synthesis of acetylcholine is decreased. 3. The activity of choline esterase was not modified by concentrations of menadione up to $1x10^{-3}$ mol. and was decreased by higher concentrations (manometric method and striated muscle). 4. Menadione increased the effect of potassium in inducing muscle contraction. 5. In high concentrations ($2.5x10^{-3}$ mol.) it induced a contraction of the striated muscle. 6. The effect of methylene blue is similar to that of menadione except that methylene blue is a potent inhibitor of the choline esterase. 7. The mechanisms of the effects of menadione are discussed in relation to its ability to react with –SH groups, to its positive oxidation-reduction potential, and to its action on the aerobic glycolysis.

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Relation of Route of Administration to Toxicity of *dl*-Serine.*

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In a previous report,[1] we have described an injurious action of *dl*-serine when administered by stomach tube. In male rats on an experimental diet, this procedure resulted in severe clinical disturbances (anorexia, loss in weight, albuminuria), demonstrable pathological lesions in the kidney, and a high mortality. It was thought that a comparison of the effects of administering the amino acid by routes other than by stomach tube might throw some light on the mechanism of the toxic action of *dl*-serine.

Experimental. Male albino rats (Rockland) weighing 95-105 g were transferred from the stock diet to an experimental diet (Diet 4), containing "Labco" vitamin-free casein 10 parts, dextrin 37, sucrose 37, Crisco 5, cod liver oil 5, "Ruffex" 2, salt mixture (Osborne and Mendel) 4.† Water was supplied *ad libitum*. After 7 days had elapsed, the daily administration of *dl*-serine by stomach tube, by intraperitoneal injection, or by admixture in the diet was initiated and continued for 14 days. The animals were maintained afterwards for an additional week on the experimental diet. A control group of rats received water by stomach tube for the same length of time. Body weight was recorded 3 times a week, and food consumption daily.

Results. Mortality is recorded in Table I and composite curves of the changes in body weight are presented in Fig. 1. Where fatalities occurred, two separate curves are shown, one for the animals dying (Curves IIA and IIIA) and one for those surviving (Curves IIB and IIIB). The data on food consumption have been omitted for the sake of brevity but the values paralleled the changes in body weight.

Control animals receiving water only by stomach tube (Group I) showed no visible ill-effects and they all survived. Thus, even in animals on a deficient diet, prolonged stomach tubing is not harmful. Both the slow growth and the failure to gain weight during the 4th week may be ascribed to the low protein content of the diet, as well as to the probable development of a B-vitamin deficiency.

In conformity with our previous results, in Group II (receiving serine by stomach tube), a sudden loss in weight and serious disturb-

* This work was aided by a grant from the John and Mary R. Markle Foundation. A preliminary report appeared in *Federation Proceedings*, March, 1944, **3**, 10.

[1] Fishman, W. H., and Artom, C., *J. Biol. Chem.*, 1942, **145**, 345.

† This diet is identical to diet 1,[1] except that the small amounts of vitamin B complex were omitted.

TABLE I.
Route of Administration of *dl*-Serine and Mortality.

Group No.	Mg of *dl*-serine supplied daily*	Route	No. of rats	No. of deaths	Mortality in %
I	None	Stomach tube	12	0	0
II	100	" "	21	13	62
III	100	Injection	16	7	44
IV	100	Admixture in diet	11	0	0
V	200	" " "	12	0	0

* Group I received 3 cc of water daily. The daily dose of serine given to group II was dissolved in 3 cc of water and that to group III in 2 cc of water.

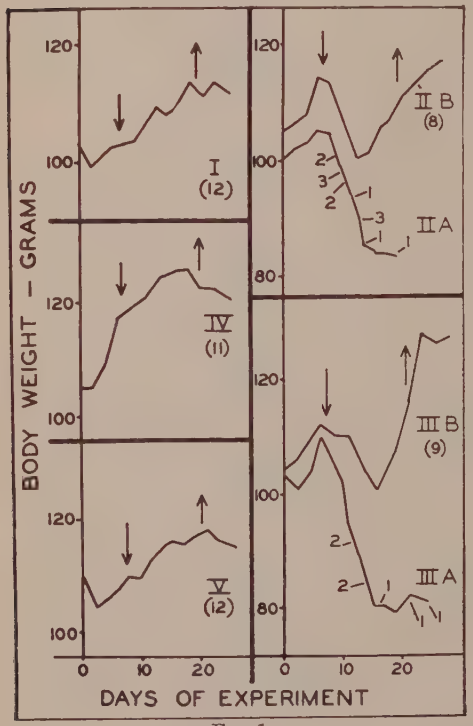

FIG. 1.

The influence of serine administration by various routes on body weight. For the periods between the arrows, the rats received either water by stomach tube (I) or *dl*-serine [by stomach tube (II); by injection (III); in the diet (IV = 100 mg daily; V = 200 mg daily)]. The number of rats surviving in each group is indicated in parentheses. The number of deaths in each day of experiment is recorded on curves IIA and IIIA.

ances were apparent during the first week of serine administration. Here it was that many deaths occurred. Later a number of these animals showed a marked recovery, in spite of the continued administration of the amino acid. The recovery was evidenced by the disappearance of clinical symptoms and by the resumption of growth. Approximately the same picture was obtained when the amino acid was injected parenterally (Group III). Here the mortality was also considerable.

When the same amount of serine was mixed in the diet (Group IV), no deaths resulted: at all times the animals appeared healthy and growth curves were very similar to those of the controls not receiving serine. Even when the amount of serine added to the diet was doubled (Group V), no ill-effects were observed.

Discussion. The same amount of *dl*-serine which is highly toxic when administered once daily either by stomach tube or by parenteral injection, is apparently harmless when it is absorbed from the diet in refracted doses during a 24-hour period. It is clear, therefore, that a sudden elevation of the level of serine in the blood and tissues is prerequisite to the production of the injury.

As for the mechanism of serine toxicity, many working hypotheses could be outlined. However, in our opinion, they may all be based on 2 general concepts. First the whole unmetabolized molecule of serine may be responsible. Thus, high concentrations of the amino acid may interfere with some essential metabolic processes by a mass action effect. This may possibly be exerted through a competitive inhibition by serine of certain enzymatic systems of the cells.

In the second interpretation, the injury may be due to products of serine metabolism. Normal intermediary products, which are present physiologically only in minimal amounts, may become injurious when they accumulate in

the tissues. Moreover, it is also possible that when the serine level is suddenly elevated, the formation of abnormal products through side reactions takes place to a large extent.‡

‡ Thus, ethanolamine, which in sufficient amounts is toxic for rats,[2] could possibly be formed by decarboxylation of serine. In this regard, the action of the intestinal flora should of course be considered. However, since serine by parenteral injection, as well as by stomach tube, is highly injurious, a role of the intestinal bacteria in the mechanism of serine injury appears unlikely.

It should be pointed out that these interpretations may apply to one or both of the components of the racemic *dl*-serine.

Summary. *dl*-Serine in 100 mg amounts daily is injurious to rats on an experimental diet when supplied by stomach tube and by parenteral injection, but is not harmful when administered mixed in the diet. Some hypotheses on the mechanism of the production of the toxic action of serine are discussed.

[2] Artom, C., and Fishman, W. H., *J. Biol. Chem.*, 1943, **148**, 423.

14768

Some Dietary Factors Which Reduce the Toxicity of *dl*-Serine in Rats.*

WILLIAM H. FISHMAN AND CAMILLO ARTOM. (Introduced by Arthur Grollman.)

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Previous data[1] point to a relationship between the diet and the severity of the injury, caused by administration of *dl*-serine. Rats on a stock diet, receiving the amino acid by stomach tube, exhibited a transient cessation of growth, and only slight ill-effects; no fatalities were observed. This contrasted with the high mortality observed under the same conditions in animals maintained on both experimental diets 1 and 2. One of these diets (Diet 2, with 30% casein) provided a very satisfactory rate of growth, almost identical with that of rats on the stock diet. It seems, therefore, that the protective action of the stock diet against serine toxicity was not due merely to the maintenance of a state of good nutrition, but rather to some specific factor, not present in sufficient amount in the experimental diets.

In this connection, casein diets are known to be relatively poor in choline, cystine, and glycine. On the other hand, the role of a deficiency of some members of the vitamin B complex could also be suspected, as the amounts of these vitamins present in our experimental diets were probably inadequate.† Accordingly, in the present study, the effect of the administration of a choline, cystine, glycine mixture, pure B vitamins, or both simultaneously, has been investigated. Special attention has been directed to the action of pyridoxine.‡

Experimental. Two experimental diets were used, diet 4[3] and diet 4 supplemented with choline HCl, glycine, and *l*-cystine (50 mg of each, daily). The daily intake of vitamins in animals on the stock diet and in animals receiving pure B vitamins is indicated in Table I. *dl*-Serine was administered by stom-

* This work was aided by a grant from the John and Mary R. Markle Foundation. Preliminary report in *Federation Proceedings*, March, 1944, **3**, 10.

[1] Fishman, W. H., and Artom, C., *J. Biol. Chem.*, 1942, **145**, 345.

† 10 g of diets 1 and 2[1] contained 100 γ nicotinic acid, 10 γ riboflavin, 10 γ thiamin HCl, 1 γ pyridoxine, 1 γ Ca pantothenate.

‡ We are indebted to Dr. E. E. Snell for having pointed out to us the possibility of pyridoxine having an action antagonistic to serine, on the basis of his then unpublished results.[2]

[2] Snell, E. E., and Guirard, B. M., *Proc. Nat. Acad. Science*, 1943, **29**, 66.

[3] Artom, C., and Fishman, W. H., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 239.

TABLE I.
Approximate Daily Intake of Individual B-vitamins.

B-vitamins	B-vitamin sol. (complete)* γ	Stock diet† γ
Pyridoxine	50	23-47
Thiamine HCl	50	54-65
Riboflavin	50	21-28
Nicotinic acid	1500	1440
Calcium Pantothenate	200	200-257
Inositol	200	Not determined
p-Aminobenzoic Acid	200	Not determined

* The pure vitamins were generously supplied by Merck and Co. The vitamin solutions were injected in a volume of 0.5 cc once daily for the first 3 weeks of experiment.

† An average daily intake of 10 g of diet was assumed in calculating these values. Data on the B-vitamin content of our stock diet (Rockland Rat diet, complete) were supplied by Arcady Farms Milling Company, Chicago, Ill.

ach tube according to the general plan of experimentation previously described.[3]

Results. Data on mortality are recorded in Table II, while composite curves of changes in body weight for most of the experimental groups are shown in Fig. 1 and 2. Separate curves are given for the animals dying (Curve VIa) and for those surviving (Curve VIb).

On the stock diet (Group I) no deaths occurred from serine administration and growth was affected only for a few days. In the animals on the experimental diets receiving serine but no B vitamins, mortality was high and the loss in weight was marked, even though supplements of choline, cystine, and glycine were given (Group II). When serine and the complete mixture of B vitamins were supplied (Group IV and V),

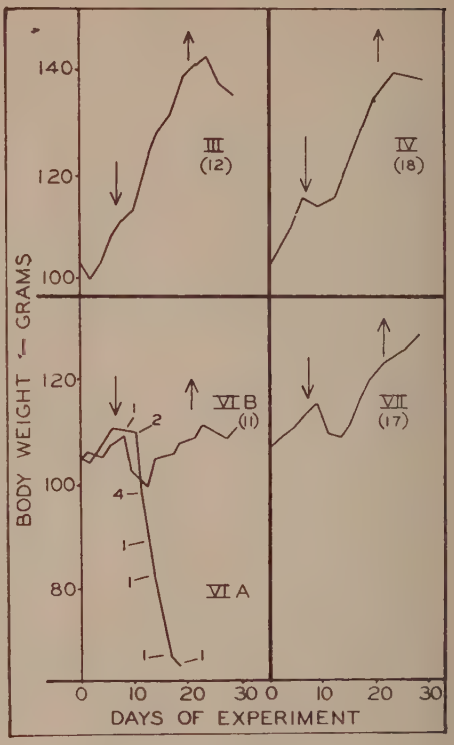

FIG. 1.

The action of serine on the body weight of rats receiving B vitamins. For the periods between the arrows the rats received by stomach tube either water (III) or *dl*-serine (other groups). All rats were on diet 4. Group III and IV received the complete B vitamin mixture; group VI, the vitamin mixture minus pyridoxine; group VII, pyridoxine only. The number of rats surviving in each group is indicated in parenthesis. For group VI the number of deaths in each day of experiment is shown on curve VIA.

TABLE II.
Effect of Dietary Factors on Mortality of Rats Receiving *dl*-Serine (100 mg daily).

Group No.	Diet	Vitamins inj.	Administered by stomach tube	No. of rats	No. of deaths	Mortality %
I	Stock	None	*dl*-serine	19	0	0
*	4	”	H_2O	12	0	0
*	4	”	*dl*-serine	21	13	62
II	4 + choline HCl, *l*-cystine, glycine	”	”	19	10	53
III	4	B complete	H_2O	12	0	0
IV	4	”	*dl*-serine	19	1	5
V	4 + choline HCl, *l*-cystine, glycine	”	”	19	1	5
VI	4	B minus Pyridoxine	”	22	11	50
VII	4	Pyridoxine (100 γ per day)	”	19	2	11

* See Groups I and II of our previous series of experiments.[3]

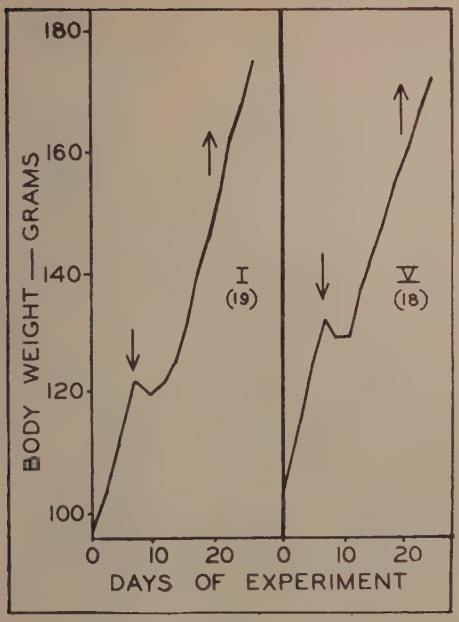

FIG. 2.

A comparison between the action of serine on the weight of rats on stock diet (I), or on diet 4, supplemented with choline, cystine, glycine and the complete B vitamin mixture (V). The number in parentheses indicates the number of rats surviving.

the mortality was very low. Growth was only slightly affected by serine administration and the animals appeared to be in good health as did those on the stock diet. Group V, on the experimental diet, supplemented with choline, cystine, and glycine and all the B vitamins, appeared to withstand the ill effects of serine best, the growth curves of these rats being almost identical to that of the animals on stock diet receiving serine (Fig. 2).

On the other hand, when pyridoxine was omitted from the B vitamin mixture (Group VI), the administration of serine produced severe clinical disturbances and many deaths resulted. The survivors did show recovery of growth in a manner very similar to that of corresponding animals receiving serine but no B vitamins.[3] Pyridoxine alone (Group VII) was able to reduce the mortality considerably; the drop in weight was less marked and the recovery more rapid than that of Group VI, receiving B vitamins without pyridoxine.

Discussion. It appears that a mixture of pure B vitamins is capable of alleviating the severity of clinical symptoms and of reducing considerably the mortality of rats receiving serine by stomach tube. As the stock diet contains adequate amounts of these vitamins, the protective action of this diet against serine injury may thus be explained. Of course, the possibility that other factors may also be involved cannot be excluded.

Of the B vitamins we have tested, pyridoxine appears to be most effective. It is difficult to state at present whether the action of this vitamin is a more general one on the nutrition of the tissues, or whether the vitamin is involved specifically in some phases of the metabolism of serine. However, pyridoxine has been shown to reverse the inhibition of growth in *Strep. lactis* caused by certain amino acids including serine.[2] In this respect, it may also be pointed out that a relationship between pyridoxine and the metabolism of proteins[4] and of tryptophane[5] has been shown previously.

Summary. The administration of ample amounts of pure B vitamins to rats on an experimental diet, receiving *dl*-serine by stomach tube, reduces considerably the mortality and the severity of the clinical symptoms. Of the B vitamins tested, pyridoxine was most effective.

[4] Lepkowsky, S., Roboz, E., and Haagen-Smit, A. J., *J. Biol. Chem.*, 1943, **149**, 195.

[5] McHenry, E. W., and Gavin, G., *J. Biol. Chem.*, 1941, **138**, 471; Foy, J. R., and Cerecedo, L. R., Abstracts, Division of Biological Chemistry, American Chemical Society, Atlantic City, Sept. 8, 1941; Voris, L., and Moore, H. P., *J. Nutrition*, 1943, **25**, 7.

14769

Effect of Streptomycin and Other Antibiotic Substances upon *Mycobacterium tuberculosis* and Related Organisms.*†

Albert Schatz and Selman A. Waksman.

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The ability of certain saprophytic organisms to inhibit the growth of *Mycobacterium tuberculosis* has long been recognized. As far back as 1885, Cantani[1] obtained favorable effects from the treatment of a tuberculous patient with a culture of a common bacterium. Later, Vaudremer,[2] using extracts of *Aspergillus fumigatus* for the treatment of patients suffering from tuberculosis, reported satisfactory results. Similar observations were reported[3] concerning the effects of extracts of fungi belonging to the *Penicillium* group. The recent progress made in our knowledge of antibiotic substances and their action upon various disease-producing bacteria suggested the advisability of studying the relation of some of these substances to the causative agent of tuberculosis and other related organisms.

Some of the antibiotic substances, like penicillin,[4] have already been shown to have no effect upon *M. tuberculosis*. Other substances, however, such as streptomycin were found[5] to inhibit the growth of both *M. tuberculosis* and *M. phlei*, the degree of sensitivity of these organisms to streptomycin being greater even than that of *Escherichia coli*. The limited toxicity of streptomycin to animals is of particular interest in connection with this problem.

A detailed comparison of the antibacterial spectra of streptomycin and of streptothricin,[6] on the one hand, and of a number of other antibiotic agents, on the other, is presented in Table I. The results illustrate the high degree of selectivity of the various antibiotic substances upon different bacteria. Of the 2 gram-negative organisms used in these experiments, *E. coli* is sensitive only to streptomycin, streptothricin, clavacin, and gliotoxin, and is not affected at all or only to a very limited extent by the other three agents. The second gram-negative organism *Pseudomonas aeruginosa* is sensitive only to streptomycin and to clavacin; it showed no sensitivity at all to the other substances, at least in the concentrations used. Of the 3 strains of mycobacteria used in these experiments, *M. phlei* is most sensitive to all compounds, whereas the avian TB is more resistant than the human strain to some of the substances, especially streptomycin and streptothricin, but not to others.

When the *E. coli* units are taken as a basis for comparison, streptomycin is found to be more than 50 times as active as streptothricin against the human strain of *M. tuberculosis* and about 5 times as active against the saprophytic *M. phlei;* it is also more active against avian TB. It may be of interest to note here that *Actinomyces griseus*, the organism that produces streptomycin, contains a second antibiotic factor. This factor is ether-soluble and is present largely in the mycelium of the organism; it is more active against the avian than against the human strain of *M.*

* Journal Series paper, New Jersey Agricultural Experiment Station, Rutgers University, Department of Microbiology.

† With partial support from a grant made by the Commonwealth Fund of New York.

1 Cantani, A., *Zentrbl. Med. Wiss.*, 1885, **23**, 513.

2 Vaudremer, A., *C. R. Soc. Biol.*, 1913, **74**, 278, 752.

3 Smith, M. I., and Emmart, E. W., *Pub. Health Repts.*, 1944, **59**, 417; Miller, D. K., and Rekate, A. C., *Science*, 1944, **100**, 172.

4 Abraham, E. P., Chain, E., Fletcher, C. M., Gardner, A. D., Heatley, N. G., Jennings, M. A., and Florey, H. W., *The Lancet*, 1941, **241**, 177.

5 Schatz, A., Bugie, E., and Waksman, S. A., Proc. Soc. Exp. Biol. and Med., 1944, **55**, 66.

6 Waksman, S. A., and Woodruff, H. B., Proc. Soc. Exp. Biol. and Med., 1942, **49**, 207; *J. Bact.*, 1943, **46**, 299. See also a recent paper by Woodruff, H. B., and Foster, J. W., Proc. Soc. Exp. Biol. and Med., 1944, **57**, 88.

TABLE I.
Antibacterial Activities of Different Antibiotic Substances.

Substance	Activity, units per gram of dry, ash-free materials*						
	Ps. aeruginosa	*E. coli*	*B. mycoides*	*B. subtilis*	*M. phlei*	Human TB (H37)	Avian TB
Streptomycin	9,500	38,000	63,000	380,000	320,000	250,000	3,800
Streptothricin	< 6,700	100,000	< 6,700	330,000	170,000	13,000	< 6,700
Chaetomin	<10,000	< 10,000	50,000,000	100,000,000	50,000,000	100,000	100,000
Fumigacin	< 3,000	3,000	300,000	590,000	150,000	22,000	15,000
Clavacin	2,000	20,000	20,000	50,000	67,000	3,300	6,700
Actinomycin	<20,000	< 20,000	>20,000,000	> 20,000,000	2,000,000	6,000,000	600,000
Gliotoxin	<20,000	30,000	1,500,000	2,000,000	1,000,000	200,000	200,000

* A unit is that amount of material that will just inhibit the growth of the organism in 1 ml of culture medium.

tuberculosis.

Because of the high toxicity of clavacin and its low activity against the human TB, it is automatically eliminated from practical consideration. Chaetomin, a substance that has a high degree of activity against both TB strains, is also eliminated from practical consideration, because of the fact that it has so far been found to have no activity *in vivo*, due to its neutralization by certain mechanisms in the body fluids.[7]

Streptomycin appears to be a promising antibiotic substance from the point of view of practical utilization against the human TB organism, because of its relatively greater *in vitro* activity against this strain of *M. tuberculosis* and its lower toxicity,[8] as compared with the very high toxicity of actinomycin,[9] as well as the relatively high toxicity of gliotoxin.[10]

Streptomycin was also found to exert a definite bactericidal action upon the human strain of *M. tuberculosis*, as illustrated by the results of the following experiment. An aqueous solution of streptomycin containing 3,000 units per 1 ml was heated at 70°C for 10 minutes. This solution was added, in concentrations of 300, 100, 30, 10, 3, 1, .3, .1 and .03 units per 1 ml, to a number of 5 ml portions of Long's liquid medium placed in tubes. All the tubes were inoculated with a clump of the H37 strain of the organism, and incubated at 37°C. Of the 4 tubes prepared for each dilution, 2 were removed after 3 days and the remaining 2 after 7 days. The TB clumps were carefully taken out of the tubes, washed in sterile portions of water, and streaked over the surface of a slant of Long's agar medium, to determine viability of the cells. Slides were also prepared from each clump of cells and stained by the acid-fast technic. The use of clumps of cells rather than of suspensions facilitated the removal of any streptomycin adsorbed on the bacterial cells that might have tended to inhibit the growth of the bacterium on the agar slants.

The 3-day exposure tests gave positive growth on all the slants from the tubes containing 300 to .3 units per 1 ml, but the number of TB colonies was fewer and slower in developing than those in the controls. This may be due to the fact that a few cells within the interior of the clump were not in direct contact with the bactericidal agent. The tubes receiving 0.1 and 0.03 units of streptomycin per 1 ml gave growth on the slants nearly equal to that on the controls, both in the number of colonies and in their size and development. The 7-day exposure tests showed that the slants inoculated from the tubes receiving 300 units of streptomycin solution gave no significant amount of growth of the TB organism. Slants from the 100 and 30 unit tubes showed a trace of growth in the form of a few isolated spots. Definite development of occasional colonies was observed on the slants corresponding to the

[7] Waksman, S. A., and Bugie, E., *J. Bact.*, 1944, in press.

[8] Jones, D., Metzger, H. J., Schatz, A., and Waksman, S. A., *Science*, 1944, **100**, 103.

[9] Waksman, S. A., Robinson, H., Metzger, H. J., and Woodruff, H. B., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 261.

[10] Robinson, H. J., Thesis, Rutgers University, 1943.

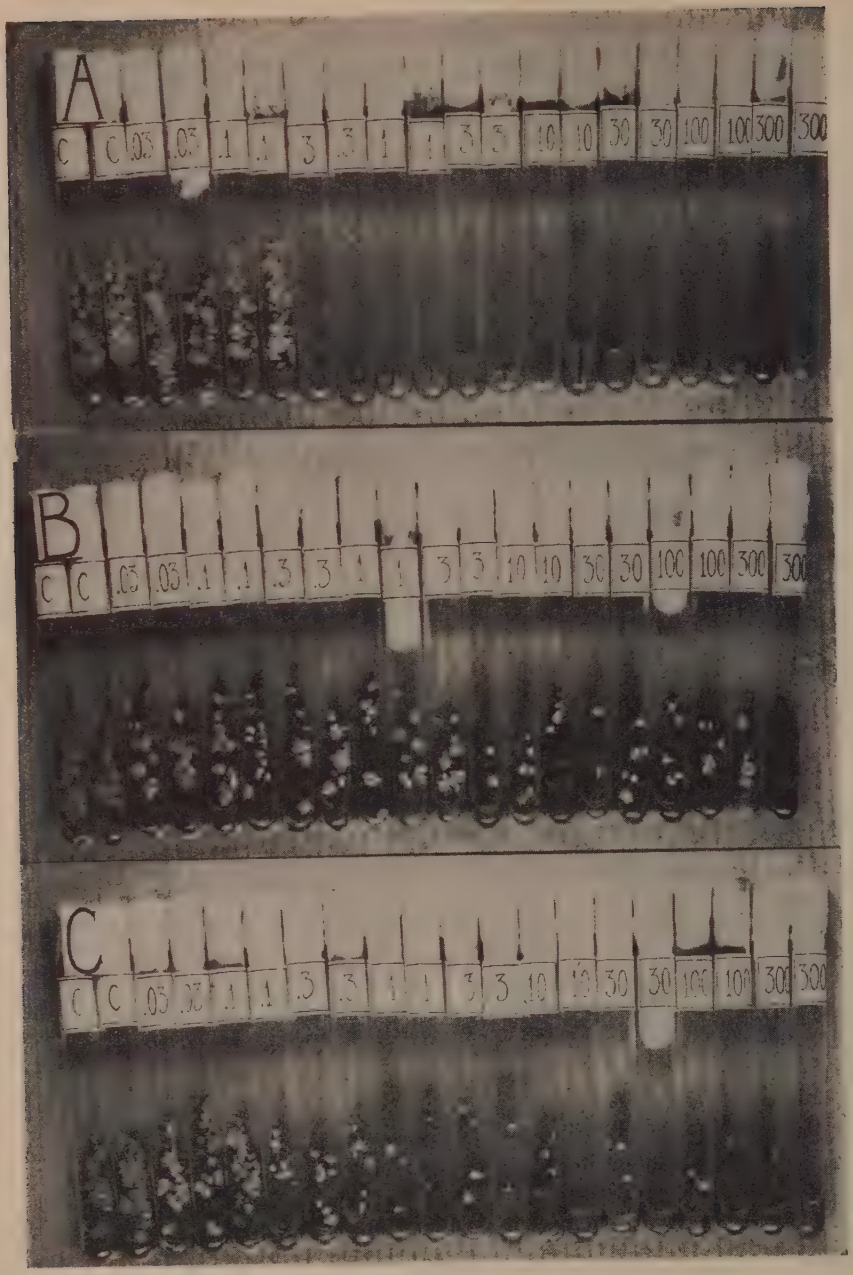

FIG. 1.

The bacteriostaic and bactericidal action of streptomycin upon a human strain (H37) of *M. tuberculosis:* the figures on the tubes give the units of streptomycin, C being the control.

A. Bacteriostatic action.
B. Bactericidal action, three days.
C. Bactericidal action, seven days.

tubes receiving 10 to 1 unit of streptomycin, whereas those slants that were prepared from the tubes with .3 to .03 units of streptomycin gave almost as good growth as the controls.

In each series of agar slants, corresponding to the two periods of exposure of the TB cells to the different concentrations of streptomycin, there was an increase in the number and size of the colonies as the dilution of the antibiotic agent, to which the clumps had originally been exposed, was increased. Approximately the same sized clumps were selected for inoculation, mashed thoroughly, and smeared over the entire surface of the slant.

A microscopic examination of the acid-fast stained slides corresponding to the 3- and 7-day exposures revealed the fact that whereas the control cultures contained cells normal in size, shape, and in staining reaction, the tubes receiving 300 units streptomycin solution showed that a few cells were somewhat longer, more slender and less intensely acid-fast; occasionally a clump appeared as a mass of cocci (round, acid-fast granular bodies), imbedded in a mass of blue substance; some of the cells appeared blue (non-acid-fast). The tubes receiving 100 to 10 units of the streptomycin solution showed that all cells were normal but some blue substance appeared in an occasional clump. The tubes with 3 to .03 units of streptomycin solution showed all the cells to be normal, without any blue substance. It has been suggested[11] that this "cyanophile" substance is indicative of subsequent disintegration or autolysis of the cells. The bacteriostatic and bactericidal effects of streptomycin upon *M. tuberculosis* are illustrated graphically in Fig. 1.

These and other results not reported here showed that the addition of 200-300 units of streptomycin per 1 ml of medium, in which living cells of the TB organism were suspended, was sufficient to kill the cells within a period of a few days; the addition of smaller amounts of streptomycin brought about the killing of the cells if a long enough period of incubation was allowed, namely, 10 or more days. The death of the cells was not accompanied by their visible disintegration, although certain atypical forms occasionally appeared. The mechanism of the action of streptomycin upon the TB cells must, therefore, be considered as still a matter for further investigation.§

It may be of interest to report here briefly the relative activity of some of the antibiotic substances against a bacterium that has frequently been classified with the Actinomycetales and that is said to be closely related to the Mycobacteria, namely, *Erysipelothrix*. *E. muriseptica* was found‡ (Table II) to be more sensitive to streptomycin than to streptothricin on the basis of their respective *E. coli* units. The practical advantage of streptomycin over clavacin and chaetomin lies again in its lower toxicity, as compared with clavacin, and in its *in vivo* activity, as compared with chaetomin.

TABLE II.
Effect of Antibiotic Substances upon *E. muriseptica*.

Substance	Activity units	Activity against *E. muriseptica*
Clavacin	100,000*	30,000
Chaetomin	1,000,000†	>100,000
Streptothricin	100,000*	15,000
Streptomycin	30,000*	12,000

* *E. coli* units.
† *B. subtilis* units.

The results of a study of the action of streptomycin upon several typical actinomycetes are presented in Table III. The organisms selected for this experiment comprise both soil organisms and animal pathogens. The results obtained show that some actinomycetes are highly sensitive to streptomycin. The pathogenic *Streptomyces* strain (*A. albus*) was inhibited by less than 1 unit of the substances, whereas the 2 pathogenic *Nocardia* strains (*A. asteroides* and *A. gypsoides*) were more resistant. There was even a greater variation among the saprophytic strains; *A. griseus*, the organism producing streptomycin, was most resistant to its action,

[11] LaPorte, R., *Ann. Inst. Past.*, 1943, **69**, 262.

§ At the suggestion of the authors the effect of *in vivo* tests of streptomycin upon *M. tuberculosis* is now being studied by Drs. Feldman, Hinshaw, and Heilman of the Mayo Foundation, Rochester, Minn.

‡ These tests were made by Miss H. C. Reilly of this laboratory.

TABLE III.
Effect of Streptomycin upon Growth of Different Actinomycetes.

Organism	Units of streptomycin required to inhibit growth in 1 ml of medium	
	2 days	3 days
*A. albus**	0.4-1.25	0.4-1.25
A. asteroides	—	12.5
A. gypsoides	—	4.0-12.5
A. antibioticus	< 0.4	< 0.4
A. lavendulae	1.25	1.25
A. griseus	>12.5	>12.5
Actinomyces 3462	4-12.5	4-12.5

* Said to be causative agent of ear infection.

whereas *A. antibioticus,* the organism that produces the powerful actinomycin, was most sensitive to it.

Summary. *Mycobacterium tuberculosis* is subject to the bacteriostatic action of a variety of antibiotic substances. There is considerable variation in this respect, both in the sensitivity of the same organism to different substances and of different species or even strains of the same species of *Mycobacterium* to the same substance. Streptomycin is also highly effective against various related organisms, namely, *Erysipelothrix* and actinomycetes, comprising both saprophytic and parasitic strains, with considerable variation among different species.

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Identification of the Serum Fraction Carrying Syphilitic Reagin by Electrophoresis.*

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In earlier reports[1,2] it was shown that the serum proteins in all stages of syphilis show characteristic and significant alterations from the normal values. An increase in all of the globulin components was found with a decrease in the concentration of the albumin component which maintained the total protein concentration in the normal range. Since no correlation could be established between the concentration of any globulin component or components and the strength of the quantitative serological reactions, the fraction containing the reagin responsible for the positive serological reactions could not be determined. The present investigations were undertaken to establish the identity of the syphilitic reagin.

Soon after the Wassermann reaction was introduced and other flocculation and complement fixation tests appeared, a number of studies were made on syphilitic sera in an effort to determine in which fraction or fractions the reagin occurred.[3-7 and others] In general all workers were in agreement on the fact that the reagin was present in the globulin fraction and was not present in the albumin fraction. Further work on fractionation of the globulins, however, brought forth disagreement among the investigators on the globulin fraction carrying the reagin. The earlier fractionation technics involved separating the globulin into pseudoglobulin and euglobulin and the lack of exact characteriza-

* This investigation was supported by a grant from the Abbott Fund of Northwestern University. The work has also been aided by a grant for equipment from the American Association for the Advancement of Science to Dr. H. B. Bull.

1 Cooper, J. A., and Atlas, D. H., in *Physical Biochemistry* by H. B. Bull, John Wiley and Sons, New York, 1943.

2 Cooper, J. A., *J. Invest. Derm.,* in press.

3 Mackie, T. J., *J. Hygiene,* 1923, **21**, 386.

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6 Noguchi, J., *J. Exp. Med.,* 1909, **11**, 84.

7 Eagle, H., *Laboratory Diagnosis of Syphilis,* C. V. Mosby Co., St. Louis, 1937, Chap. XVI.

TABLE I.
Effect of Inactivation and Flocculation by the Kahn Antigen on Syphilitic Sera.

Patient	pH	A / G	α / A	β / A	γ / A	Remarks
G.B.	7.81	1.26	0.11	0.25	0.43	Untreated
	7.80	1.28	0.11	0.25	0.41	Inactivated
	7.82	1.51	0.11	0.27	0.29	" and flocculated
J.M.	8.60	0.80	0.15	0.56	0.55	Untreated
	8.60	0.76	0.16	0.58	0.58	Inactivated
	8.60	0.90	0.14	0.52	0.48	" and flocculated

tion of the fractions led to divergent results.

In the work to be reported here, salt fractionation was employed, but the electrophoretic components present in the fractions thus obtained were determined and a correlation established between serological reactions and the electrophoretic globulin components present in the sample.

Methods. Electrophoretic Methods. The 0.025 M lithium diethyl barbiturate, 0.025 M diethyl barbituric acid, 0.025 M lithium chloride buffer of Longsworth, Shedlovsky, and MacInnes[8] was used in all cases with the exception of the serum from J.M. shown in Table I. In this analysis the 0.1 M sodium diethyl barbiturate, 0.02 M diethyl barituric acid buffer[9] was used. The latter buffer has the advantage that the δ and ϵ boundaries separate from the γ boundaries.[9] The samples of serum and the protein fractions were diluted 1:2 with the buffer before dialysis. The cell was the conventional 3-piece cell of Tiselius[10] and the lens system was that of Philpot as modified by Svensson.[11]

Fractionation with ammonium sulfate. In order to identify the electrophoretic fraction which carries the Kahn and Wassermann reagin, samples of syphilitic serum giving strong Kahn titers, were combined to give a pooled sample of 1000 ml.

The pooled serum was diluted with an equal volume of distilled water and 2 liters of saturated ammonium sulfate solution were added slowly by a capillary pipette while stirring the solution. The precipitated globulins were filtered off and the filtrate was brought to 0.75 saturation with ammonium sulfate. The precipitate (A) was filtered off.

The precipitated globulins were dissolved in a liter of 0.9% sodium chloride and the resulting solution filtered. Saturated ammonium sulfate solution was added through the capillary pipette to 0.22 saturation and the precipitate filtered off (GI). The filtrate was brought to 0.34 saturated and the precipitate (GII) filtered off. The filtrate from GII was brought to 0.40 saturation with ammonium sulfate and the insoluble fraction (GIII) filtered off. The remainder of the protein (GIV) was precipitated at half saturation with ammonium sulfate. In the fractionation procedures, no attempt was made to control the pH.

All of the fractions were dissolved in 0.95 sodium chloride solution to give a final concentration around 5 g of protein/100 ml. These solutions were dialyzed in the ice box against 0.9% NaCl until free of sulfate ion. The protein concentrations were determined by Kjeldahl analyses.

Serological Reactions. The various fractions were submitted for serological examination by the standard Kahn test, the quantitative Kahn test, the Wassermann (Kolmer) test, the Kahn temperature verification test,[12] the Kahn salt verification test,[12] and the Hinton test. All of the tests with the exception of the Wassermann (Kolmer) reaction were done on both inactivated samples and uninactivated samples.†

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[9] Longsworth, L. G., *Chem. Rev.*, 1942, **30**, 323.

[10] Svensson, H., *J. Biol. Chem.*, 1941, **139**, 805.

[11] Svensson, H., *Kolloid Ztschr.*, 1940, **90**, 141.

[12] Kahn, R. L., *J. Lab. and Clin. Med.*, 1943, **28**, 1170.

† The author wishes to express his thanks to Dr. Herman N. Bundesen, President of the Chicago Board of Health, for making serum from clinical

Results. In an attempt to determine the fraction or fractions of syphilitic serum which combine with the Kahn antigen to produce the flocculus a large sample of blood was obtained from each of two patients with strongly positive serological reactions and clinical manifestations of syphilis. The serum was prepared and divided into 3 portions. An electrophoretic analysis was made on one portion without further treatment. The second sample was inactivated according to the standard Kahn technic for 30 minutes at 56°C. After inactivation an electrophoretic analysis was obtained. The third sample was inactivated by heating 30 minutes at 56°C and Kahn antigen was added to the undiluted serum to obtain a copious floc. After standing overnight in the ice-box, the floc was centrifuged off and an electrophoretic analysis was made on the supernatant fluid.

The results obtained are given in Table I. No significant change in the distribution of electrophoretic fractions of the sera resulted from inactivation. The treatment of inactivated serum with the Kahn antigen produced a decrease in the γ/A ratio in both cases. The decrease in this fraction in the serum of J.M. was smaller. These experiments indicate that the reagin is present in the γ globulin fraction of syphilitic sera.

Another approach to the problem was made. A large volume of syphilitic serum (1000 ml) was obtained by pooling small quantities of serums which gave strongly positive Kahn reactions. This serum was fractionated as outlined above with ammonium sulfate and five fractions were obtained.

The original pooled serum and the 5 fractions were analyzed electrophoretically. The number of components present in each fraction was determined and the concentration of each fraction obtained. The electrophoretic

TABLE II.
Electrophoretic Analyses of Protein Fractions Obtained from Syphilitic Serum by Ammonium Sulfate Precipitation.

Fraction	Fractional sat. with $(NH_4)_2SO_4$ causing precipitation	pH	Potential gradient volts/cm in tube	Total protein conc. g/100 cc	Concentration of Electrophoretic fractions, g/100 cc				Mobilities of electrophoretic components, cm/sec volt $\times 10^5$			
					Albumin	α Glob.	β Glob.	γ Glob.	Albumin	α Glob.	β Glob.	γ Glob.
GI	0.22	7.80	7.31	6.00	—	—	1.64	4.36	—	—	3.1	0.4
GII	0.34	7.81	7.31	6.21	—	0.59	2.24	3.38	—	4.9	3.5	0.3
GIII	0.40	7.80	7.31	4.61	—	2.66	1.02	0.93	—	4.6	3.4	0.3
GIV	0.50	7.80	7.31	2.59	—	2.59	—	—	—	4.9	—	—
A	0.75	7.80	7.31	5.50	4.30	0.60	0.60	—	6.1	4.6	3.1	—
Original serum		7.80	7.31	7.20	3.60	0.76	1.12	1.63	5.9	4.1	2.9	0.2

material available; to Dr. John White, Director of Laboratories, Chicago Board of Health; H. Worley Kendell, Surgeon (R), U.S.P.H.S., medical director of the Chicago Intensive Treatment Center; Miss Elsie Miller, Chief Laboratory Technician, Chicago, Intensive Treatment Center; Dr. A J. Shaughnessy, Director of Laboratories, Chicago Branch, Illinois State Department of Health, for their kind cooperation in running the serological reactions.

components were identified by their mobilities.

The results of the electrophoretic analyses are given in Table II.

Samples of the 5 fractions were submitted for serological examination. The results are given in Table III.

Consideration of the results of the electrophoretic analyses and the results of the standard Kahn test shows that all of the fractions which give positive serological reactions contained β and γ globulins. Since fraction GI, which gave the highest titer in the quantitative Kahn reaction contains no α globulin, the latter fraction apparently carries none of the reagin. These findings indicate that the Kahn reagin is carried in the β or γ globulin fraction or both. The Wassermann (Kolmer) reaction was strongly positive in the fractions having positive Kahn reactions. The reagin for the complement fixation test is carried in the same fractions as the Kahn reagin.

Coburn and Moore[13] report that the Wassermann reagin present in the sera of patients with lupus erythematosus who were apparently not syphilitic, is found in the β_2 and γ fractions. The present work would then indicate that the true reagin and the reagin in falsely positive blood are present in the same fractions. The fact that their electrophoretic mobilities are the same, however, does not mean that they are alike in other respects.

All fractions which gave positive Kahn reactions also gave positive verification reactions in both the temperature and salt dispersability tests, and hence no indication of a non-syphilitic reagin was found. Fraction GIII which showed a negative Kahn reaction when inactivated, nevertheless gave a positive verification test after inactivation. The inactivated samples of this fraction gave positive Kahn reactions and verification tests. These phenomena are probably due to the small amounts of γ globulin present in this fraction.

The Hinton test was negative for all fractions except GIII. In the test of this fraction without inactivation the result was doubtful. The presence of 0.9% NaCl in the samples may have interfered with the technic of the examination with this reaction. The possi-

[13] Coburn, A. F., and Moore, D. H., *Bull. Johns Hopkins Hosp.*, 1943, **73**, 196.

TABLE III.
Serological Reactions of Protein Fractions from Syphilitic Sera.

Fraction	Electrophoretic fractions present	Standard Kahn		Quant. Kahn	Temperature verification		Salt verification		Wassermann	Hinton	
		Uninact.	Inact.	Inact.	Uninact.	Inact.	Uninact.	Inact.	Inact.	Uninact.	Inact.
Original sera	A, α, β and γ	pos.	pos.	40	—	—	—	—	—	—	—
GI	β and γ	,,	,,	120	syph.	syph.	syph.	syph.		neg.	neg.
GII	α, β and γ	,,	,,	80	,,	,,	,,	,,		,,	,,
GIII	α, β and γ	neg.	,,	—	,,	,,	,,	,,	—	,,	doubt.
GIV	α	,,	neg.	—	neg.	neg.	—	—	—	,,	neg.
A	A, α and β	,,	,,	—	,,	,,	—	—	—	,,	,,

bility that the mechanism for this reaction differs from that of the Kahn and Wassermann reactions also exists.

Summary. 1. Electrophoretic analyses are reported for syphilitic sera, before inactivation by heating to 56°C for 30 minutes, after inactivation, and after inactivation and flocculation with the Kahn antigen. No significant change occurred on inactivation but flocculation with the antigen apparently removes some γ globulin from solution. 2. Electrophoretic analyses and serological reactions of protein fractions obtained by salting out with ammonium sulfate are reported. From the data obtained, the β or γ globulin fractions or both have been identified as carriers of the Kahn and Wassermann reagins.

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Physiology of Bacteria-free *Trichomonas vaginalis*. VII: Temperature in Relation to Survival and Generation Time.*

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Information regarding the response of *Trichomonas vaginalis* to various temperatures should be useful in studies of the epidemiology of the infection and may indicate the limitations of heat in therapy.

The effect of temperature extremes upon *Trichomonas vaginalis* in contaminated cultures has been described. Leuckardt[1] and Dock[2] reported a sensitivity to cold. Fukushima[3] subcultured the organisms successfully after 3 days in an ice box. Fischer[4] reported growth following 21 days storage at 3-5° C. Mohr[5] described multiplication of trichomonads that had been frozen for 24 hours after being kept for 8 days at room temperature. Survival at room temperature for 7 days was reported by Fukushima,[3] and for 8 days by Mohr.[5] Weiler[6] failed to subculture from cultures kept at 18-20° C for 36 to 48 hours. The thermal death time was described as 10 minutes at 46°C by Davis[7] and 5 minutes at 47° C by Matsuda.[8]

In the present studies, a culture-medium containing cysteine, peptone, liver infusion, maltose and human serum was used.[9] Test organisms were obtained from 2-day cultures, demonstrated to be bacteria-free with thioglycollate medium. The numbers of organisms in the inocula were determined by hemocytometer count. Fluctuations in temperature were minimized by immersing the culture tubes in water. Temperatures were maintained within less than $\pm$ 0.5° C. Thermometers were standardized against a Bureau of Standards certified instrument under conditions comparable to the test involved. Experimental temperatures below the prevailing

* Aided by a grant from the Ortho Research Foundation.

1 Leuckardt, R., *Die Parasiten des Menchen und die von ihnen herruhrenden krankheiten*, 2 aulf. Bd. 1, pp. 311-317, Leipzig, E. F. Winter, 1879.

2 Dock, G., *Tr. Path. Soc. Phila.*, 1896, **17**, 287.

3 Fukushima, K., *Nihon Fujinka Gk. Z.*, 1934, **29**, 8 (*Jap. J. M. Sc., Gnec.*, Feb., 1936).

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room temperatures were obtained in an incubator which was placed in a refrigeration room.

Survival at reduced temperatures was checked by maintaining 15 to 20 inoculated cultures at the experimental temperature. At intervals duplicate cultures were removed to an incubator at 37° C and examined for evidence of multiplication after appropriate periods of incubation. Cultures showing no gross evidence of multiplication were incubated for a maximum of 9 days. The results of duplicate and triplicate tests are summarized in Table I.

TABLE I.
Survival of *Trichomonas vaginalis* at Reduced Temperatures.

Temp. °C	Hours survival
1 to 4	96-144
24	120-150
25	154
26	Multiplication occurs

Exposure to a temperature of approximately —72° C was accomplished by pipetting 0.2 ml of inoculated culture fluid into each of a series of sterile cotton-stoppered tubes, which were placed in a bath of solid carbon dioxide in 95% alcohol. Several tubes were transferred to a 37° C water bath as soon as the solid state was reached. The remainder were removed at one minute intervals. After melting the culture fluid and warming to 37° C, 0.1 ml was transferred to C.P.L.M. medium and incubated at 37° C for a minimum of 9 days with periodic examination for multiplying organisms. The remainder of the melted culture fluid was examined microscopically for motile trichomonads. The cultures exposed to —72° C revealed no motile organisms and no growth in the subcultures when the exposure had been for one minute or longer. Merely solidifying the fluid in the freezing mixture did not destroy the majority of the organisms though large vacuoles were observed in the cytoplasm.

The thermal death time was investigated by allowing 10 cotton-stoppered culture tubes containing 4.0 ml of culture fluid to reach the experimental temperature in a water bath. They were then inoculated at thirty-second intervals with 0.2 ml of a 2-day-old culture having a population of about 2000 organisms per cubic millimeter. Subcultures were made at intervals up to 30 minutes. In this way 10 duplicate subcultures were obtained containing organisms exposed for a given interval. The procedure was similar to that previously described for testing the action of antiseptics.[9] In order to investigate exposures of less than 5 minutes the number of culture tubes exposed was correspondingly reduced so that the allotted exposure time might elapse between the inoculation of the first tube and the first transfer to a subculture. For example, in an exposure time of 4 minutes, 8 tubes were inoculated at 30 second intervals. Thirty seconds following the inoculation of the eighth tube, the first tube inoculated contained organisms which had been exposed for 4 minutes. The subcultures were then incubated and examined at intervals of several days for evidence of multiplication. The death time at each temperature investigated is shown in Table II.

TABLE II.
Thermal Death Time of *Trichomonas vaginalis.*

	Minutes exposure							
Temp. °C	2	3	4	5	10	15	20	30
45				+	+	+	+	+
45.8				+	+	+	+	+
47				+	+	+	—	—
48				+	+	—	—	—
49				+	—	—	—	—
50	+	+	—	—	—	—	—	—

+ = Survival.
— = No survival.

Response to intermediate temperatures was measured in terms of generation time. In this investigation, 8 ml of sterile culture fluid were pipetted into each of a series of 10 sterile cotton-stoppered tubes and 2 ml of sterile, undiluted serum, adjusted to pH 6, were added. A 2-day-old culture supplied the inoculum. The population of this culture was determined by averaging 4 hemocytometer counts and was adjusted if necessary by dilution with sterile culture fluid. The number of organisms used in the inoculation was reduced in those cultures which were incubated at temperatures where the rate of multiplication was high. This was done in order that

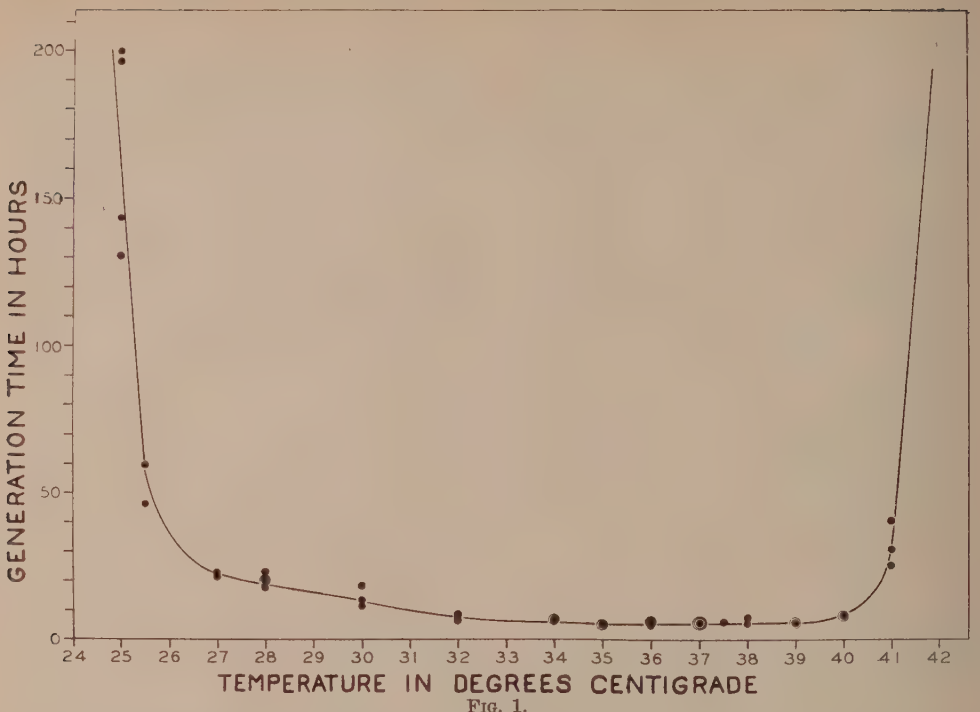

FIG. 1.

the final population might be determined before the cultures had reached the phase in which metabolic wastes limited further increase in population. The volume of culture fluid was maintained at a constant level during the period of incubation by replacing the cotton with sterile cork stoppers to prevent evaporation. After approximately 40 hours in the incubator, the cultures were brought to room temperature and the population determined by a hemocytometer. The generation time (g) was calculated from the equation, $g = \frac{t}{3.3 \log b/B}$, where t is the time in hours, b is the population in organisms per cubic millimeter at time t, and B is the original population as determined from the inoculation. This equation is derived from $g = \frac{t \log 2}{\log b - \log B}$.[10] The curve plotted in Fig. 1 summarizes the data from these experiments. It will be noted that at 25° C the generation time in half the experiments exceeded the maximum survival time for cultures exposed to this temperature. It is therefore concluded that 25° C is the lowest temperature at which the organisms multiply. The upper limit was 42° C and the minimum time required for optimal cell division, 5 to 7 hours, obtained over a broad range from 34° to 39°C.

Conclusions. 1. *Trichomonas vaginalis* survives exposure at temperatures near the freezing point for 96 to 144 hours and at 24-25°C for 120 to 154 hours. Exposure to –72°C for one minute destroys the organisms. 2. The thermal death time for temperatures above 43°C has been recorded. At 50°C it is 4 minutes. 3. The lowest temperature consistent with multiplication is 25°C; the upper limit 41°C. 4. The minimum time required for cell division is 5 to 8 hours and was observed over a broad range from 34° to 39°C.

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14772

Phthalates and Vitamin K.

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Recent publications[1-5] have suggested that vitamin K activity is due to the eventual conversion of the natural vitamins K_1 and K_2, as well as their synthetic analogues, to phthalic acid or related compounds. According to this theory phthalic acid is regarded as the true biological carrier of vitamin K activity. The evidence for this belief consists largely of the following: (1) reported observations that the highly active 2-methyl-1, 4-naphthoquinone (menadione) and the potassium salt of 2-methyl-1, 4-naphthoquinone-3-sulfonate can be transformed into phthalic acid under mild conditions such as might prevail in the body; and (2) experimental evidence of a slight anti-hemorrhagic activity of phthalic acid and a much higher activity of diethyl phthalate. However, another investigator[6] could find no evidence of vitamin K activity from the oral administration of 100 mg of potassium acid phthalate or 23 mg of diethyl phthalate, as tested in 300 g vitamin K-deficient chicks. In view of this disagreement in the literature, there may be reported the following investigation on the possible vitamin K activity of several phthalate esters.

Day-old chicks were fed a vitamin K-deficient diet of the heated grain type,[7] so that hypoprothrombinemia, as indicated by increased blood coagulation time in several test animals, resulted in 13 to 15 days. The various test substances were administered orally by syringe in 0.1 cc doses. After 18 hours the chicks were bled by decapitation, and blood coagulation time for each chick was determined at room temperature on duplicate samples of approximately 1 cc of blood collected in small vials. The phthalates were of purified chemical grade (Eastman). Cod liver oil (U.S.P.) was used as the solvent in the first experiment and ethyl laurate (Eastman) in the second.

In Table I are given the results of both experiments. A coagulation time of less than 6 minutes was regarded as a positive response for vitamin K activity; in these experiments all of the chicks which received menadione actually had blood coagulation times of ½ to 4 minutes, usually 1 to 2 minutes. A coagulation time of 6 minutes or greater was regarded as a negative response; the coagulation times actually observed in the negative cases were usually above 20 minutes, almost one-half remaining uncoagulated after 1 hour.

As may be seen in the table, no vitamin K activity was shown by dipropyl, di-isopropyl, or dibutyl phthalate at a level of 10 mg per chick, or by diethyl phthalate at levels of 0.1, 10, or 100 mg per chick. On the other hand, a positive response was regularly elicited by 0.01 mg (10γ) or 0.001 mg (1γ) of menadione. Therefore, if diethyl phthalate had any vitamin K activity under the conditions of these experiments, it must have been less than one-hundred-thousandth the potency of menadione. This result is in agreement with the findings of Dam[6] in larger chicks. It must be noted that the previous reports of the vitamin K activity of diethyl phthalate were apparently based on rat experiments in which hypoprothrombinemia was induced by ligation of the bile duct. Since a different species

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2 Shemiakin, M. M., Schukina, L. A., and Shvezov, J. B., *Nature*, 1943, **151**, 585.

3 Bochvar, D. A., Schukina, L. A., Chernyshev, A. S., Semenov, N. G., and Shemiakin, M. M., *J. Am. Chem. Soc.*, 1943, **65**, 2162.

4 Shemiakin, M. M., Schukina, L. A., and Shvezov, J. B., *J. Am. Chem. Soc.*, 1943, **65**, 2164.

5 Shemiakin, M. M., Shchukina, L. A., and Shvetzov, Yu. B., *J. Gen. Chem.* (U. S. S. R.), 1943, **13**, 398.

6 Dam, H., *Nature*, 1943, **152**, 355.

7 Ansbacher, S., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 421.

TABLE I.
Vitamin K Activity as Determined by Oral Administration to Chicks.

	Group	No. chicks	Supplement			Vitamin K activity —	Vitamin K activity +
Exp. I	1	4	Cod liver oil	0.1	cc	4	0
	2	5	Diethyl phthalate	0.1	mg	5	0
	3	5	" "	10.0	"	5	0
	4	4	Dipropyl "	10.0	"	4	0
	5	4	Di-isopropyl "	10.0	"	4	0
	6	4	Dibutyl "	10.0	"	4	0
	7	5	Menadione	0.01	"	0	5
Exp. II	1	5	None			4	1*
	2	6	Ethyl laurate	0.1	cc	6	0
	3	8	Diethyl phthalate	10.0	mg	8	0
	4	9	" "	100.0	"	9	0
	5	8	Menadione	0.001	"	0	8
	6	8	"	0.01	"	0	8
	7	7	Diethyl phthalate + menadione	0.1 0.001	" "	0	7

* Small chick; had not eaten well.

and a different technic were used in the experiments herein reported, the two investigations are not directly comparable. However, it may be pointed out that oral administration to chicks is the generally accepted method of testing for vitamin K activity. After the completion of these experiments a publication was received in which it was reported[8] that neither intravenous sodium phthalate nor oral diethyl phthalate increased the prothrombin in a jaundiced man who responded readily to intravenous injection of the phosphoric acid ester of 2-methyl-1, 4-naphthoquinone.

In a recent discussion of the hypoprothrombinemia produced by indandione derivatives,[9] it was stated that the indandione compounds might degrade to phthalic acid. In this connection may be mentioned the anti-vitamin K or anti-prothrombin test made with diethyl phthalate (Expt. II, group 7). This group of vitamin K-deficient chicks was fed 0.1 mg (100γ) of diethyl phthalate in ethyl laurate, followed about 15 minutes later by 0.001 mg (1γ) of menadione. As may be seen in Table I, diethyl phthalate did not prevent the positive response for vitamin K activity from this small dose of menadione under these experimental conditions.

Summary. When tested by oral administration to baby chicks, no vitamin K activity was observed for dipropyl, di-isopropyl, or dibutyl phthalate at a level of 10 mg per chick, or for diethyl phthalate in doses as high as 100 mg per chick. These results differ from those reported in some rat experiments, in which diethyl phthalate was considered active. When tested by a single dose method in depleted chicks, diethyl phthalate did not show anti-vitamin K activity.

[8] Karrer, P., and Koller, F., *Helvet. Chim. Acta*, 1943, **26**, 2114.

[9] Kabat, H., Stohlman, E. F., and Smith, M. I., *J. Pharm. Exp. Therap.*, 1944, **80**, 160.

14773

An Apparatus for Nebulizing Liquids.*

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The apparatus described below was designed† especially for the atomization of human sera. It was employed by the Commission on Influenza during studies concerning the prophylactic effects of the inhalation of convalescent human sera against experimental influenza infections in man.[1] Because of its excellent efficiency in providing a large output of nebulized liquid a detailed account of its construction seems worth while.

The nebulizing unit (Fig. 1) consists of a container and a metal atomizer. The container may be made either of metal or glass and be of any size or shape. That shown in Fig. 1 was designed to hold a large amount of liquid (500 cc). It was made by fusing an outlet of 22 mm glass tubing into the bottom of a short-necked, round-bottom, pyrex flask of one liter capacity.

The atomizer is constructed of chromed brass tubing. Fig. 2 (3 sectional views) shows various aspects of its design. It consists of a

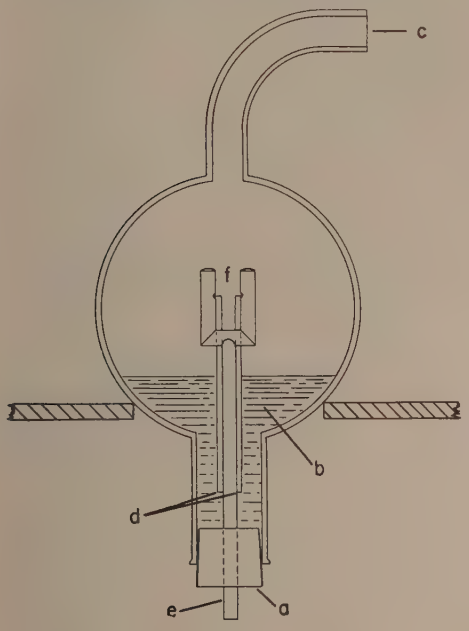

FIG. 1

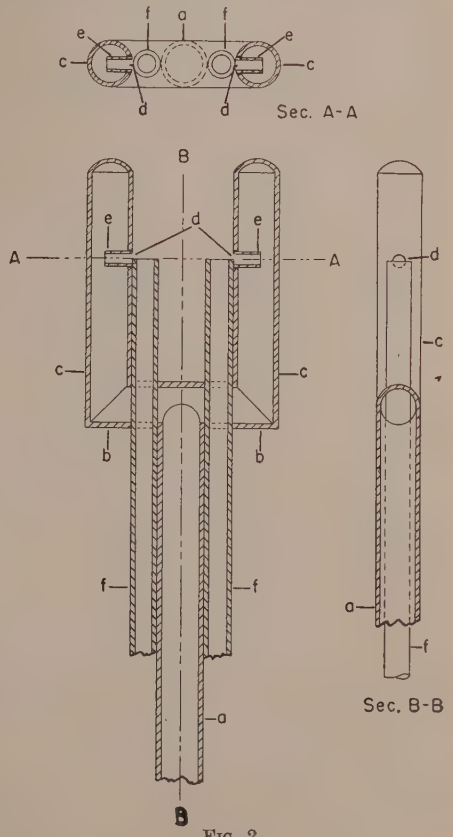

FIG. 2.

* This investigation was supported by the Influenza Commission, Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the Army, Preventive Medicine Service, Office of the Surgeon General, U. S. Army.

† Mr. D. H. Loosli designed and built the metal nebulizer.

single inlet tube (Fig. 2-a) 6 inches long with a 3/16 inch inside diameter which communicates with a "U" shaped head or nozzle, made of tubing of similar size. The base (Fig. 2-b) of the nozzle is one inch wide and the stems (Fig. 2-c) one inch each in length. The stems are closed at their ends and on their side walls directly opposed discharge orifices (Fig. 2-d) slightly less than 1/16 inch in diameter are made. The orifices are made by soldering at right angles short pieces of 1/8 inch tubing (Fig. 2-e) into the walls of the nozzle stems to give a jet effect to the outgoing air. Two smaller tubes through which the fluid flows (Fig. 2-f) 4 inches in length with 1/8 inch outside and 3/32 inch inside diameters and opened at both ends are placed parallel to the stems of the nozzle. They extend through the base of the nozzle downward and adjacent to the air inlet tube. The upper ends of the liquid-bearing tubes (Fig. 2-f) lie approximately in the plane of the centers of the discharge orifices (Fig. 2-d). The joints where the tubes (Fig. 2-f) pass through the base (Fig. 2-b) of the nozzle are soldered to render them air tight.

The unit is assembled by inserting the metal atomizer into the neck of the inverted container where it is held in place by a rubber cork (Fig. 1-a). It is then adjusted so that the ends of the tubes (Fig. 1-d) are about ¼ inch from the cork. The serum or liquid (Fig. 1-b) to be nebulized is added through the outlet opening (Fig. 1-c) of the container in a sufficient quantity (25 to 500 cc) to immerse the serum tube opening (Fig. 1-d). The main air tube (Fig. 1-e) is then connected to an air supply (compressed air etc.) and under pressure, air is forced through it and out through the 2 opposing air jets (Fig. 1-f) in the nozzle. As the jets of air cross the upper ends of the tubes containing fluid (Fig. 2-f) an aspirating effect is produced in them and liquid is drawn up into the respective air streams. The streams moving at a high velocity in directly opposite directions, meet head on at approximately the medium plane between the stems of the nozzle, and reduce the liquid to a state of fine suspension.

This is shown by the fact that the droplets leaving the container appear as a fine mist which does not settle but disseminates through the room air. In spite of the marked turbulence of air in the container the liquid is only slightly agitated when the nebulizer is in operation. The bombardment of the 2 opposing jets of liquid and air directly against one another at close proximation, which creates the effect of a baffle, is considered to be the important and new feature of the nebulizer.

The output of liquid from this nebulizer will depend on the size of the outlet opening of the container and the rate of air flow through it. Using serum (undiluted) and an air flow of 1.1 cubic feet per minute the output was approximately one cc per minute. When four 22 mm outlets were made in the same container and 2.5 cubic feet of air passed through it the serum output was approximately 3.5 cc per minute. The unit has also been used satisfactorily in atomizing influenza virus in suspensions of mouse lungs during studies on the survival of influenza virus in the air. The details of the practical use to which this nebulizer was put in influenza studies will be reported elsewhere.[1]

[1] Commission on Influenza, to be published.

14774 P

Studies on Certain Filtrable Viruses. VII. Antigenic Properties of Entire Embryo Fowl Pox Vaccine.

D. L. Kerlin and Robert Graham. (Introduced by F. W. Tanner.)

From the Department of Animal Pathology and Hygiene, University of Illinois.

In a previous report,[1] the results of artificial exposure of chickens from farm flocks inoculated with fowl pox vaccine prepared from entire chick embryo were described. The vaccine consisted of a composite of the chorio-allantoic membrane, yolk, yolk sac, albumen, embryo proper, and fluids from inoculated embryos that showed gross lesions on the chorio-allantoic membrane. Thirty-five healthy farm flocks composed of 25,164 chickens were inoculated by the 3-stick method with the desiccated vaccine suspended 1-100 in tryptone broth. Observation of over 6,000 of the birds nine days after inoculation showed 95.85% with positive take reactions. The report on results of artificial exposure indicated that 95.77% of 71 birds taken from 4 of the flocks showed immunity to fowl pox virus approximately one year after vaccination.

The present note concerns an additional observation on the duration of immunity produced by entire embryo vaccine. One hundred and seventy-nine birds culled at 4 different intervals from one of the flocks approximately 2 years subsequent to vaccination were artificially exposed to fowl pox virus. A 1-100 suspension of entire embryo virus in tryptone broth was inoculated by the 3-stick method into the patagium, the virus being of the same strain as that employed in vaccination. The exposed chickens were examined at two consecutive weekly intervals for lesions of fowl pox following exposure. Results of observations are presented in Table I.

Of the 179 birds artificially exposed two years following vaccination, six susceptible and two with doubtful reactions were encountered. The 19 nonvaccinated control birds exposed to the challenging virus proved susceptible. Though the number of birds included in this observation is small in comparison with the original number of birds vaccinated, it appears that a large proportion, 96.68%, proved immune to fowl pox virus exposure 2 years after vaccination. The percentage of immunity compares favorably with 95.77% found one year subsequent to vaccination.

TABLE I.
Artificial Exposure of Chickens Vaccinated Two Years Previously with Entire Embryo Fowl Pox Vaccine.

Date of Exposure	No. exposed*		No. susceptible†		% immune	
	Vaccinated	Control	Vaccinated	Control	Vaccinated	Control
5-1-44	24	6	0	6	100	0
6-14	47	3	0	3	100	0
7-25	86	6	4	6	95.81	0
10-3	22	4	2	4	90.90	0
Total	179	19	6	19	96.68	0

* Entire embryo fowl pox virus by 3-stick method.
† Birds showing fowl pox lesions, doubtful reactions considered negative.

1 Kerlin, D. L., and Graham, Robert, Proc. Soc. Exp. Biol. and Med., 1944, **55**, 225.

14775

Prolonged Viability of Yellow Fever Virus in Serum Mixtures Containing Ammonium Sulfate.

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Because viruses tend to die out rather rapidly in liquid media if no growing cells are present, it is necessary to hasten all operations as much as possible while the virus under study is in this circumstance. When the experiments here described were performed little had been recorded about what constitutes the most favorable medium for yellow fever virus *in vitro*.[1-3] It was well known that simple saline solutions are rapidly lethal; and also that the presence of serum proteins exerts a profound preservative effect. However, under the best conditions the serum-saline mixtures are not especially effective. These considerations, together with the surmise that intracellular media are slightly acidic, led to trials with various inorganic salts which in the main tend to create a slightly acidic reaction. Following is a summary of what was found when ammonium sulfate was used.

Mouse brains infected with neurotropic yellow fever virus were ground and diluted with saline so that the final mixture contained a 10^{-2} dilution of mouse brain. Three mixtures were then prepared.

	I	II	III
Normal human serum	0.5 cc	0.5 cc	0.5 cc
10% ammonium sulfate	1.5 "	0.5 "	0.0 "
0.8% sodium chloride	2.5 "	3.5 "	4.0 "
Virus suspension 10–2	0.5 "	0.5 "	0.5 "

Mixture No. I contains 3% ammonium sulfate; No. II contains 1%; and No. III contains none. The final strength of mouse-brain suspension is 10^{-3}. No. III represents the control, which is a mixture commonly employed in routine experiments with yellow fever virus. All 3 mixtures were incubated at 37° C continuously; material for test inoculations was removed daily under sterile precautions. Test animals were standard white mice; each received 0.03 cc. of the inoculum intracerebrally in the manner which is standard for this work. The measure of viability of the virus was its capacity to cause illness and death in mice between the fourth and tenth days after intracerebral injection.

The control mixture (III) has lost much of its living virus after 48 hours of incubation, and practically all after 3 days. The mixture containing 3% ammonium sulfate in addition (I) shows a greater viability. The best exhibition is by the mixture containing one per cent ammonium sulfate; it retained much living virus through 6 days of continuous incubation, and appreciable amounts for 7 and 8 days.

Additional experiments established the fact that the optimal concentration of ammonium sulfate lies in the neighborhood of 1%. Another series of experiments showed that much benefit arises from a shift of the pH in the medium. When a buffered phosphate mixture of pH 6.3-6.5 is added instead of ammonium sulfate, a similar, though apparently not quite so great, prolongation of life of the virus is shown. The reactions of the ammonium sulfate-serum-virus mixtures soon after preparation were: (I) pH 6.2; (II) pH 6.3; (III) pH 7.4. After 5 days of incubation, the pH of each was 6.2, 6.5, and 7.7, respectively.

When one per cent ammonium sulfate mixtures containing virus and serum similar to the ones described are kept at room temperatures (22-25° C) and shielded from light, the capacity to kill mice in the usual fashion is maintained for as long as 20 days. Here again, the viability of virus in ammonium sulfate solutions is much longer than in normal serum dilutions alone.

[1] Lewis, P. A., *J. Exp. Med.*, 1930, **52**, 113.

[2] Milam, D. F., *Am. J. Trop. Med.*, 1939, **19**, 297.

[3] Frobisher, M., (a) *Am. J. Trop. Med.*, 1931, **11**, 127; (b) *Am. J. Hyg.*, 1930, **11**, 300; (c) *J. Exp. Med.*, 1931, **54**, 733.

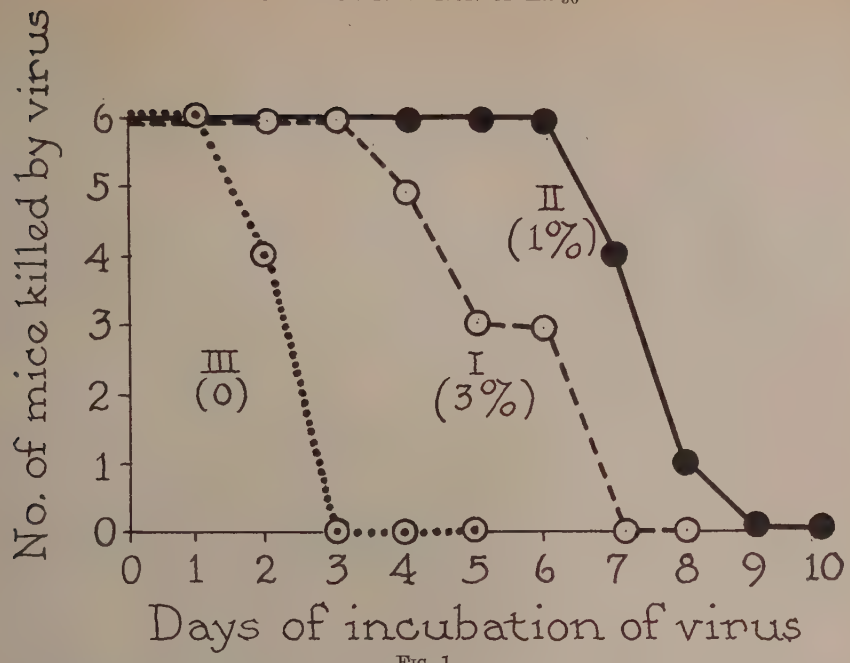

FIG. 1.

Experiment of September 17, 1941. Six mice were injected intracerebrally each day with each mixture. The graphs show the number of mice which died between the fourth and tenth days after injection.

Conclusions. (1) Neurotropic yellow fever virus in serum-saline dilution is protected by the addition of one per cent ammonium sulfate. (2) Much of the preservative effect is due to a shift of the reaction of the medium to the acid side. The optimal reaction appears to be near pH 6.3-6.5. (3) Addition of ammonium sulfate to 1% concentration seems to be the most convenient method of bringing about an optimal reaction.

14776

Estimation of the ED_{50} and Its Error by Means of Logarithmic-Probit Graph Paper.*

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With the increased emphasis on evaluation of all-or-none data there is need for a sound approximate procedure for arriving at a numerical expression of effect such as is represented by the ED_{50} (the dose affecting 50% of the group treated) and its standard error. The publication of Bliss[1] on the maximum likelihood solution of data based upon small numbers of subjects afforded a rigorous although somewhat tedious procedure which has been generally accepted. Using the latter for comparison, Irwin and Cheeseman[2] con-

1 Bliss, C. I., *Quart. J. Pharm. Pharmacol.*, 1938, **11**, 192.

2 Irwin, J. O., and Cheeseman, E. A., *Suppl. J. Roy. Stat. Soc.*, 1939, **6**, 174.

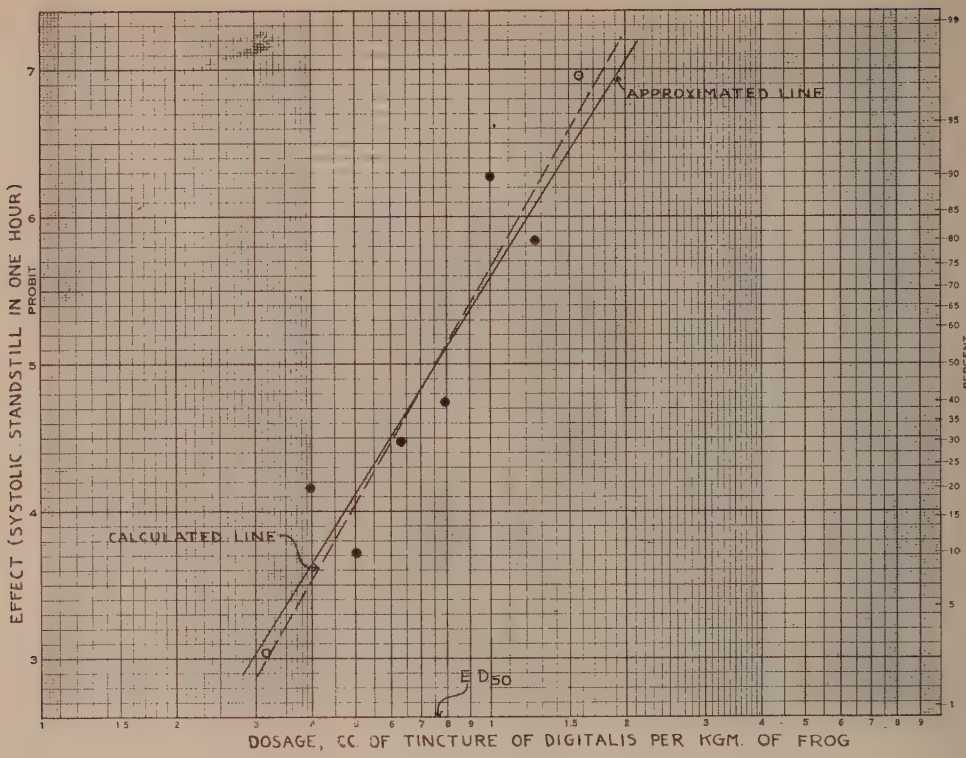

FIGURE 1

Graphic Estimation of the ED_{50} by the One-hour Frog Method.

Group No.	Dose, cc/kg	Effect P	Effect Probit
1	3.16	0/10	(3.04)
2	3.98	2/10	4.16
3	5.01	1/10	3.72
4	6.31	3/10	4.48
5	7.94	4/10	4.75
6	10.00	9/10	6.28
7	12.59	8/10	5.84
8	15.85	10/10	(6.96)

Probit $4 \cong 4.75$

$6 \cong 12.20$

$2s = 12.20 - 4.75 = 7.45$

$$\text{s.e.}_{ED_{50}} = \frac{2s}{\sqrt{2N'}} = \frac{7.45}{\sqrt{2 \times 60}} = \pm 0.68$$

cluded the Kärber method was preferable to the other short-cut methods available at that time. More recently, Litchfield and Fertig[3] have advanced a graphic procedure having much merit, particularly since it provides for the first time a simple reliable means of deriving the standard error of the ED_{50}. The technic proposed here is a simplification and extension of their method.

A logarithmic-probit graph paper (Fig. 1) has been designed to facilitate the estimations.† The paper is 11 x 16½ inches which provides space enough to plot most experi-

[3] Litchfield, J. T., Jr., and Fertig, J. W., *Bull. Johns Hopkins Hosp.*, 1941, **69**, 276.

† This graph paper may be obtained from the Special Chemicals Division, Winthrop Chemical Company, Inc., 170 Varick St., New York 13, N.Y.

mental data on a scale that permits reading the values from the graph with adequate accuracy. The vertical ruling of the paper represents a two-cycle logarithmic scale, whereas the ordinate or probit scale is divided into units of 0.02 extending from 2.60 to 7.40 probits, with heavy lines indicating the main subdivisions. Percentages are indicated on the right-hand ordinate margin, which may be used by those unfamiliar with the probit transformation. A generous margin has been allowed for tabulating the basic data. As may be noted, the range extends slightly beyond the 1% and 99% points, which is quite adequate for most biologic purposes.

In use, the original data, such as those illustrated in Fig. 1, are plotted on the paper either as the percentage effect or as the probit effect obtained by reference to a suitable table‡ without calculating the percentage. Usually each series of toxicological data includes at least one dose low enough so that all subjects survive and one high enough so that none survive. There is no finite probit value corresponding to either result. As a close approximation, Bartlett[4] suggests writing ¼ for 0 in the result obtained with the highest dose showing $0/n$, n being the number in the group on that dose.§ Similarly $n - ¼$ is substituted in the numerator of the result obtained with the lowest dose showing no survivors. This suggestion has been followed in Fig. 1, the probits for the substituted values being in parentheses and the plotted circles left unshaded to indicate the substitution. The line best fitting the points is drawn in by eye with the aid of a transparent straightedge. The estimated ED_{50} is the dosage value at 50% (probit 5.0) and is read directly from the graph paper in original dosage units.

In drawing the best-fitting line a closer approach to the maximum likelihood solution may be reached by bearing in mind that the points farthest from 50% (probit 5.0) have the least weight per animal in fixing the true position of the line.[1,3] Thus with equal numbers of experimental animals, points at probits 4.0 and 6.0 have only approximately two-thirds as much weight as one at probit 5.0 while those at probits 3.0 and 7.0 have only one-fifth as much weight. Allowance for this fact can be made in planning the experiment by increasing the size of the groups receiving the low and high doses, if desirable, or in fitting the line when the data have been plotted.

In estimating the standard error of the ED_{50} the only 2 additional values required may be read directly from the graph, *i.e.*, the dosages corresponding to 16% and 84% (probits 4.0 and 6.0). Subtracting the former from the latter gives $2\,s$, which is the estimated increment in dosage necessary to increase the effects by two probits in this dosage range. The approximate average standard error is given by the formula

$$\text{Approximate s.e.}_{ED_{50}} = \frac{s}{\sqrt{N'/2}} = \frac{2s}{\sqrt{2N'}}$$

the latter form being more convenient in the present case. In this formula, N' has a slightly different meaning from that assigned by Litchfield and Fertig, since it is the total number of animals in the groups which, from the best-fitting line, would be *expected to show effects* between 6.7 and 93.3% (probits 3.50 and 6.50). In the example given, this takes in groups 2 to 7, inclusive, and $N' = 60$. Occasionally this definition results in there being included in N' one or two groups in which the observed effects are either below 6.7% or above 93.3%. This occurs when the data on all the rest of the doses indicate that if a large number of subjects were used, a result between 6.7 and 93.3% would normally be expected. The reasons for using the expected rather than the observed result in this connection have been given in detail by Bliss.[1] The estimation of the ED_{50} and the calculation of the standard error are clearly illus-

‡ A table giving the probits corresponding to all possible results in groups up to 25 animals is available upon request from the authors; for a complete probit table see Reference 1 or Fisher, R. A., and Yates, M. A., *Statistical Tables for Biological, Agricultural, and Medical Research*, 1938, Oliver & Boyd, London.

[4] Bartlett, M. S., *Suppl. J. Roy. Stat. Soc.*, 1937, **4**, 137.

§ The authors are indebted to Dr. Chester I. Bliss for calling their attention to this reference and for other helpful suggestions.

trated in Fig. 1 from which the values of 7.55 ± 0.68 cc of digitalis tincture/kg of frog are obtained. The corresponding values by the more exact method of maximum likelihood were calculated to be 7.55 ± 0.58 and the equation of the best-fitting line with dosages (X) in logarithmic units, $Y = 5.289X + 0.356$. The line drawn in by eye coincides well with that calculated (the broken line of the figure).

It happens in this example that the graphically determined ED_{50}, obtained in less than 10 minutes, agrees exceedingly well with the maximum likelihood solution arrived at independently after the expenditure of considerably longer time, even with the aid of an automatic calculating machine. Differences between the two procedures will rarely be of practical significance. In this example the standard error is somewhat larger by the graphic procedure due principally to the fact that the line drawn by eye was slightly flatter than the best-fitting line found by the least-squares calculation. While a difference in the estimated standard error of this magnitude would scarcely modify the pharmacologic inferences, it nevertheless emphasizes the approximate nature of the graphic estimation and points up the fact that in crucial cases the standard maximum likelihood solution should be used.

Summary. A log-probit graph paper is described by which a simple graphic estimation of the ED_{50} and its standard error may be quickly made.

14777 P

Cumulative Effects of Repeated Head Trauma of Minimal Intensity.* Observations on Experimental Animals.

C. G. TEDESCHI.

From the Laboratories, Medfield State Hospital, Harding, Mass.

This investigation is concerned with the immediate and delayed effects of repeated, minimal, blunt impacts on the head.

Using as traumatizing agent an apparatus which permitted the strength of the blow to be measured exactly, a mass of 453.6 g dropping with a velocity at the point of impact of 330.9 cm/sec. was found to be just insufficient to produce loss of consciousness or any other immediate or delayed ill effects in a normal animal (subconcussive trauma). It was this blow of minimum intensity that was used in the investigation of cumulative effects of repeated trauma.

One hundred twenty rats of the same strain and of approximately the same weight were used in the experiment. Of these, 60 were given 15 blows in close succession, one minute apart; the other 60 were given the same amount of trauma (15 blows) at longer intervals, from 2 to 6 days apart, in a 50-day period. Half of each group were struck on the stationary head, and the other half were made to strike the rapidly accelerated head against a stationary object.

In all experiments precautions were taken that the head of the animal should be struck at the same region each time and that in each of the two mechanisms all animals should receive a trauma of the same momentum at all times.

Observations. Cumulative effects, as revealed by temporary loss of consciousness, at times by death or by persisting changes in behavior, were clearly shown in all groups of animals as a result of the repeated trauma. The frequency with which ill effects were displayed in the different classes at the end of the experimental period is shown in Table I.

In the great majority of cases there was a certain correspondence between the effects of the trauma during life and the extent of

* Read at the May meeting, 1944, of the Massachusetts Psychiatric Society.

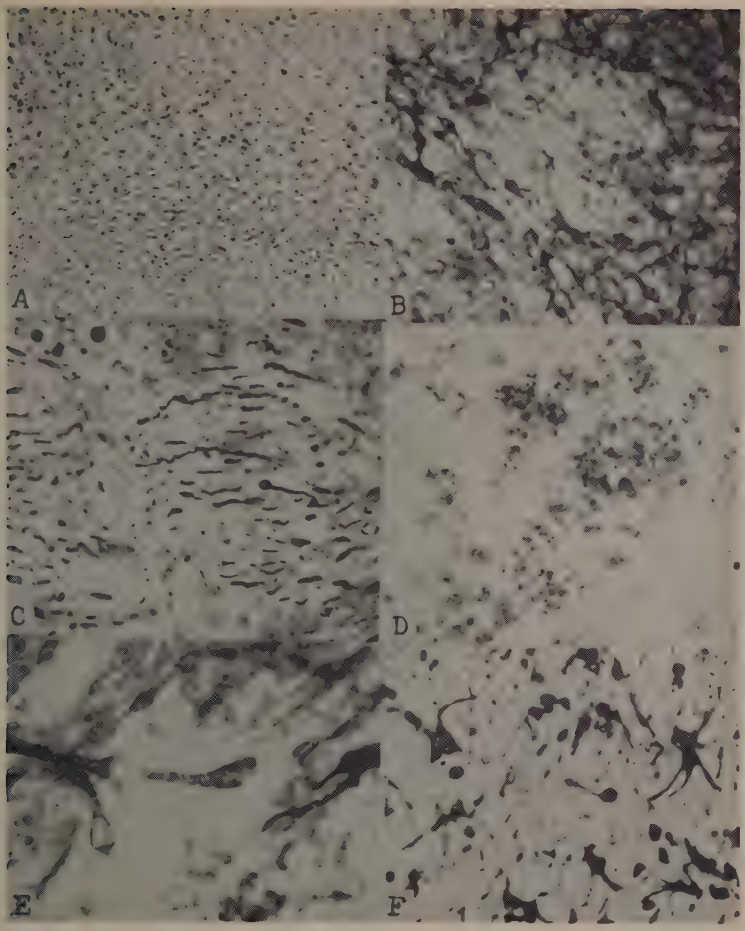

FIG. 1.

Microphotographs from the brain of a rat submitted to repeated subconcussive head trauma.

A. Obscuring of stratification, shrinkage and pyknosis of nerve cells in the cerebral cortex.

B. Disintegration with incipient reabsorption of myelin.

C. Breaking down of axons with bulbs of retraction at the ends of the fragmented fibers.

D. Scavenger cells, containing fat droplets, in an area of advanced demyelination.

E. Moniliform swelling of myelin sheaths, and club-like formations at the ends of degenerated portions of interrupted fibers.

F. Reaction of glial cells in an area with severe neuronal damage.

cerebral dramage, which appeared to be minimum in the animals clinically unaffected, and progressively more severe in the animals made unconscious, developing persisting changes in behavior, or dying as a result of the repeated trauma.

Hemorrhage or any other evidence of damage that could be detected with the naked eye was seen in only 7 out of 120 animals. Changes in neurons, affecting in a scattered fashion both nerve cells and nerve fibers, were, on the contrary, consistent microscopic

TABLE I.
Immediate and Late Effects of Repeated Subconcussive Trauma.

Procedure	15 blows	Unconscious (2″ to 60′) %	Persistent changes in behavior %	Death within 29 days %	No apparent effects %
Moving head against fixed object	1 min apart	73	13	17	27
	2-6 days apart	57	7	17	43
Moving object against fixed head	1 min apart	40	10	7	60
	2-6 days apart	40	10	0	60

findings. Obscuring of stratification, shrinkage and pyknosis of nerve cells of the cortex, and demyelinated areas were frequently found, and where demyelination and breaking down of axons were still in progress scavenger cells, loaded with fat droplets, were noticeable. Evidence of reaction of glia cells in the parts that were damaged was encountered at times, mostly without appreciable increase of glial fibers.

Summary. Rats submitted to repeated blunt impacts on the head, the strength of each of which was insufficient to cause any immediate or late ill effects, frequently revealed cumulative effects as shown by unconsciousness, at times followed by death or by persisting changes in behavior. A certain correspondence was found between the response to the trauma during life and the amount and degree of cerebral damage at post mortem examination, which consisted mainly of degenerative changes in neurons detected by present histological methods.

14778 P

Microscopic Neuronal Injury in Rats Submitted to Concussive Head Trauma Under Different Mechanisms.

C. G. TEDESCHI.

From the Laboratories, Medfield State Hospital, Harding, Mass.

Preliminary experiments in an investigation on the effects of repeated minimal impacts on the head led to the study of a number of brains of animals (68) recovering from a short period of unconsciousness after receiving a single concussive blow. Using a trauma of the same momentum (mass 567 g, velocity 345 cm/sec), half of the animals were made to strike the moving head against a stationary object and the other half were struck by a moving object on the stationary head. Although no differences were seen in the immediate effects in the two series (all animals being made unconscious by the blow for a period ranging from 10 seconds to 15 minutes), in view of the possibility of different morphological patterns resulting from the two different mechanisms of trauma, a comparative study of this material seemed of value.

Observations. At postmortem examination, no evidence of skull fracture was found in any case, and in all but one, in which petechial hemorrhages were seen in both the pons and the bulbar region, the gross inspection of the brains and even the study of the hematoxylin-eosin stained sections failed to reveal any remarkable change, except for an occasional vascular engorgement and for some doubtful signs of edema of the brain matter. However, when the stains proper for the nervous system elements were applied, definite neuronal changes were seen. Regardless of the mechanism of trauma these changes had a wide distribution, involving the cerebral

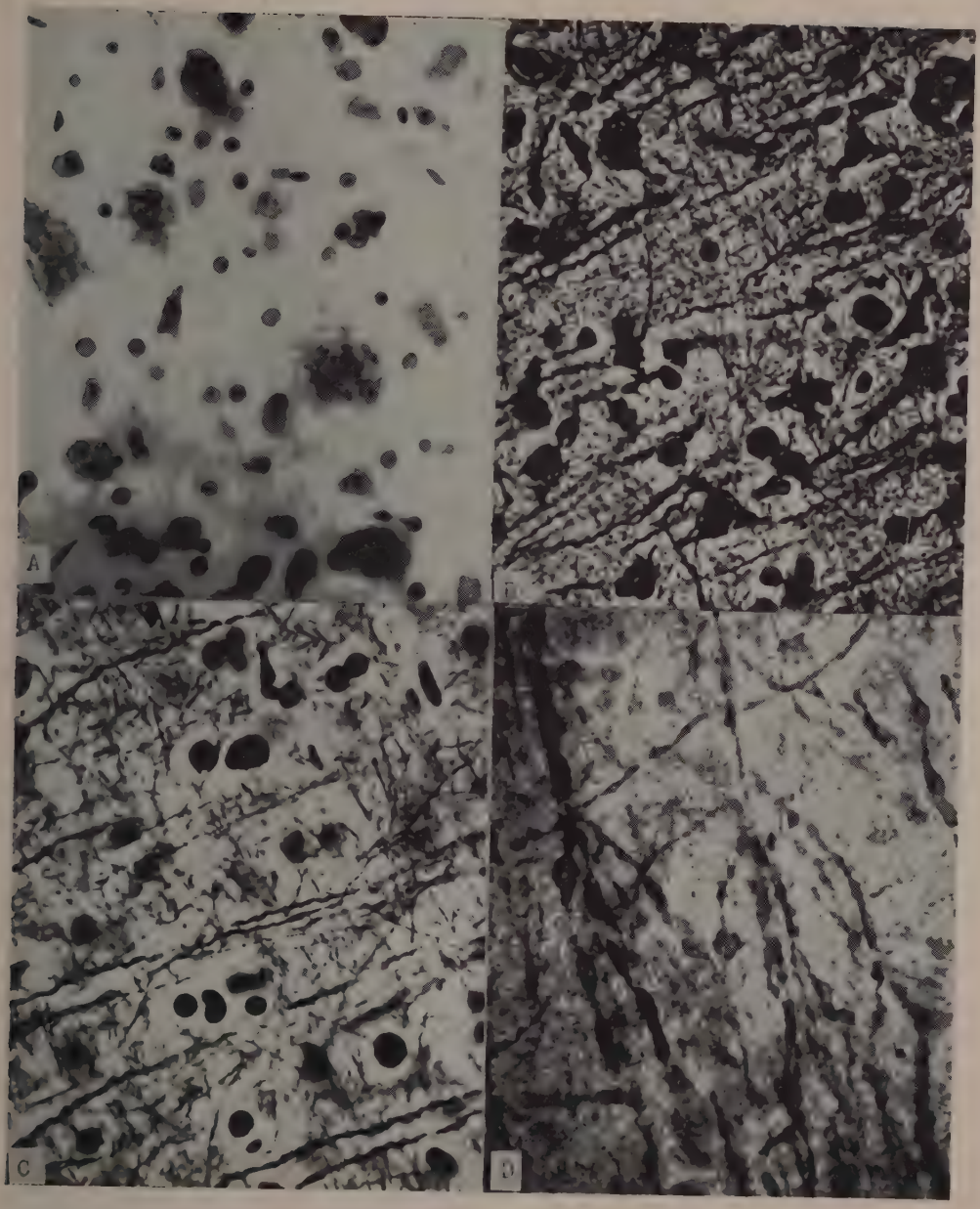

FIG. 1.

Microphotographs of the cerebrum of a rat recovering from a short period of unconsciousness induced by a blow on the head which had left skull and brain apparently intact.

A. Cortical nerve cells showing marked tigrolysis and peripheral vacuolization.

B. Thickening, spiroid course, and twisting of cortical axons.

C. Swelling, cork screw appearance, and fragmentation of medullated nerve fibers.

D. Moniliform swelling and incipient disintegration of myelin sheaths.

hemispheres, mid-brain, pons, medulla oblongata, and occasionally even the upper cervical cord, the impacts being constantly delivered on the occipito-parietal region.

In the Cajal stained preparations, thickening, spiroid course, and at times definite twisting of the axons of the cortical cells were already apparent in the animals killed at the 1st hour. The corresponding nerve cells in the Nissl stain showed obscuring of the cell outlines, tigrolysis, and huge peripheral vacuoli. These changes were not diffuse, as groups of damaged neurons alternated with others displaying normal patterns. The same type of focal change was displayed by the medullated axons which appeared poorly impregnated and swollen, with a corresponding moniliform swelling of the myelin sheaths in the Spielmeyer preparations.

More advanced myelin disintegration and neurolysis were found in the animals killed from the 4th to the 72nd hour, as shown by fragmentation of axis cylinders, with incipient neurolysis and myelin reabsorption and resolution into round, oval or irregularly shaped fat globules.

Of 16 animals killed after 2 months observation, only 4 revealed a definite pathology, characterized by focal areas of demyelination, and rarefaction of nerve cells in the cerebral cortex with obliteration of axis cylinders and glial proliferation in the most damaged areas.

Summary. Rats recovering from a temporary period of unconsciousness, varying from 10 seconds to 15 minutes, following a blunt impact on the head which had left skull and brain apparently intact, frequently revealed microscopic evidence of neuronal injury affecting, in multiple areas, nerve cells, axis cylinders, and myelin sheaths. Using a trauma of the same strength, no differences were seen, either in the immediate effects or in the type and extent of cerebral changes, when the impact was delivered on the stationary head or on the head in rapidly accelerated motion.

14779

Gastric Mucosal Lesions in Rats Submitted to Head Trauma.

C. G. TEDESCHI.

From the Laboratories, Medfield State Hospital, Harding, Mass.

A relationship between cerebral birth injury and hemorrhagic erosions in the mucosa of the alimentary tract (stomach, esophagus, first portion of the duodenum) has been known to exist for many years; yet it was not until 1932 when Cushing[1] reported additional instances of ulcers developing in individuals submitted to surgical intervention in the brain that the conception of the neurogenic origin of mucosal lesions in the intestinal tract gained widespread interest (Opper and Zimmerman,[2] Vanzant and Brown,[3] Ivy,[4] Keller[5]). Although some observations on the experimental animal have shown that these mucosal defects have a tendency to heal promptly, yet in the opinion of others this does not seem to be invariably the case, leaving unsettled the problem of the relationship between head trauma and peptic ulcer.

Rats submitted for other investigative purposes to blunt impacts on the head, single or repeated, of different intensity, revealed evidence of gastric damage in a number of cases, 14 out of 183, or 7% approximately. Some of the rats had been struck by a moving object on the stationary head and others had

[1] Cushing, H., *Surg., Gynec. and Obst.*, 1932, **55**, 1.

[2] Opper, L., and Zimmerman, H. M., *Yale J. Biol. and Med.*, 1938, **11**, 49.

[3] Vanzant, F. R., and Brown, J. A., *Am. J. Digest. Dis.*, 1938, **5**, 11.

[4] Ivy, A. C., *Arch. Int. Med.*, 1920, **25**, 6.

[5] Keller, A. D., *Arch. Path.*, 1936, **21**, 137, 165.

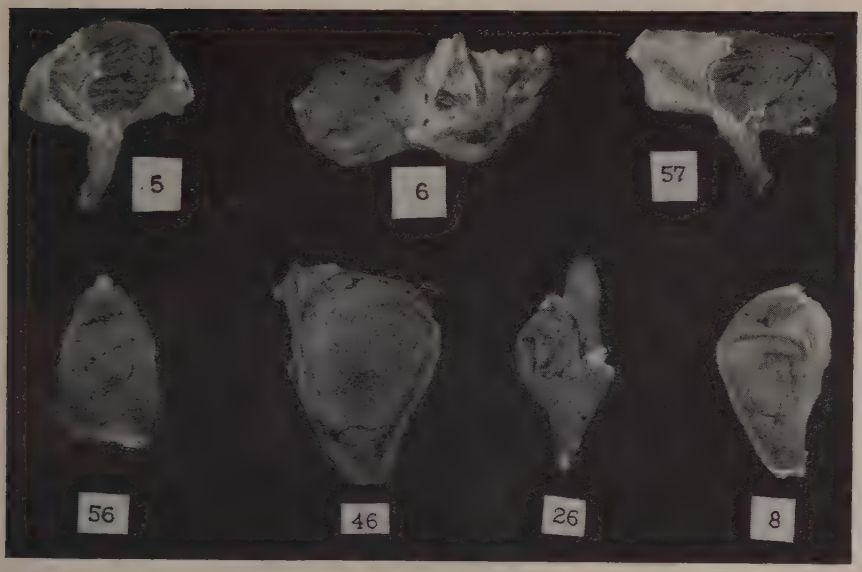

FIG. 1.
Photographs, nearly natural size, of the stomachs of rats submitted to head trauma, showing mucosal hemorrhagic erosions.

been made to strike the rapidly accelerated head against a stationary object, under controlled experimental conditions in both procedures. In 3 out of the 14 cases in which evidence of gastric damage was found the animals had received a single lethal impact; all others had been submitted to repeated "subconcussive blows" which were delivered either in close succession, 1 minute apart, or at intervals of from 2 to 6 days. By "subconcussive blow" is meant one the strength of which is insufficient to cause any apparent immediate or late ill effects in a normal animal. As is already described in another report, unconsciousness, at times followed by persistent changes in behavior or by death, was shown with a certain frequency as a cumulative effect of the repeated blows.

As displayed in the specimens shown in Fig. 1, the evidence of damage in the gastric mucosa consisted mostly of a dissemination of tiny hemorrhages which showed a tendency to align themselves along the crests of the gastric plicae. The hemorrhages were in general superficial, and as the mucosa was not depressed or otherwise altered, the resulting pattern, in most of the cases, was that of a simple hemorrhagic change; definite erosions in the hemorrhagic areas were seen less frequently, and they did not seem to penetrate deeper than the mucosal layer. Formation of erosions without concomitant evidence of hemorrhage was found in one case only (Rat 46), but there were also tiny petechiae in between the erosions and 2 larger hemorrhages could be seen, one at the cardiac and the other at the pyloric region. With the exception of 2 cases (Rats 5 and 6), in which hemorrhages were found both in the stomach and in the prestomach, most of the animals showed the hemorrhages to be confined to the stomach proper, all portions of which seemed to be equally affected. The microscopic picture in the damaged areas was that of small foci of superficial necrobiosis, with obliteration of normal mucosal patterns and widespread infiltration of recently extravasated hematic cells. The blood capillaries, small arteries and veins all showed evidence of marked engorgement, but formation of thrombi was not noticed in any case. There was a certain correlation between the occurrence of the gastric changes on one side, the severity of the trauma and correspondingly of the cerebral damage on the

other. All the animals but 2 were made unconscious one or more times during the course of the experiments and died shortly after as an immediate consequence of the trauma. In one case, (rat 6), the brain damage consisted of extensive contused lacerations, in another (rat 8) a subarachnoidal hemorrhage was found, while in a third (rat 46) multiple petechiae of the ring type were seen irregularly scattered both in the cortex and in the subjacent white matter of the cerebral hemispheres. Various stages of a fundamentally identical neurolytic change affecting, in a disseminated fashion, both nerve cells and nerve fibers, were displayed in almost all of the remaining animals, by present histological methods. If it is true that vagal, sympathetic and parasympathetic stimuli of central origin, (Cushing[1]), are responsible for the development of the gastric changes together with more striking disturbances in the circulatory system, the collateral findings of acute pulmonary edema and of petechiae in the serous membranes in 6 out of the 14 animals are significant, emphasizng the role of the neurocirculatory disturbances elicited by mechanical brain injury. Of these 6 animals, one died 5 minutes after the trauma, another after 8 minutes. These were the earliest casualties showing evidence of gastric damage. Most of the animals displaying a similar finding died between the 1st and the 7th day, and no hemorrhages or erosions were found in the rats dying or killed beyond the 29th day (the majority of the animals). These observations, in agreement with the findings of others, support the opinion most generally accepted (Moritz[6]) that if recovery from the head injury occurs and the acute effects of the gastric changes are survived, the tendency of the latter is toward prompt and complete healing.

Summary. Hemorrhagic changes, less often erosions, in the gastric mucosa were found in a number of rats submitted to blunt impacts on the head, single or repeated, of different intensities and according to different mechanisms. A certain correlation was seen between the severity of the trauma, correspondingly of the cerebral damage, and the occurrence of the gastric changes, which, when the acute effects of the trauma were survived, showed a tendency towards prompt and complete healing.

6 Moritz, A. R., *The Pathology of Trauma*, Lea & Febiger, Phila., 1942.

14780

Experiments on the Sensitizing Properties of Penicillin.

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In the clinical application of penicillin toxic reactions are not very common. Nevertheless, reactions have been observed which are at least suggestive of an allergic manifestation. Keefer and associates[1] stated that in the treatment of 500 cases with penicillin toxic effects were rare, though occasional chills with fever, headache, and flushing of the face, muscle pain and constriction of the chest, and urticaria have been noted. Cohn[2] reported no toxic effects in a series of 48 cases, while Dawson and Hobby[3] observed occasional instances of various reactions in a series of 100 cases. Usually the toxic reactions were of a mild nature. Urticaria was recorded in 3 cases. In discussing toxic reactions from penicillin, Lyons[4] noted urticaria with fever

1 Keefer, C. S., Blake, F. G., Marshall, E. K., Jr., Lockwood, J. S., and Wood, W. B., *J. A. M. A.*, 1943, **122**, 1217.

2 Cohn, A., Studdiford, W. E., and Greenstein, I., *J. A. M. A.*, 1944, **124**, 1124.

3 Dawson, M. H., and Hobby, G. L., *J. A. M. A.*, 1944, **124**, 611.

TABLE I.
Anaphylactic Response to Intravenous or Intracardiac Reinjection of Penicillin. All Animals Also Sensitized to Horse Serum.

No.	Sensitizing dose, units		Incubation period, days	Shocking dose, units	Result	Shocking dose of horse serum, cc	Result
1	1 x 300 + 1 x 400 + 1 x 500	i.p.	30	5000 i.v.	No symptoms	0.5	Died in 3 min.
2	1 x 300 + 1 x 400 + 3 x 500	"	30	8350 i.c.	Died in 5 min.		
3	"	"	31	21400 "	Symptoms with recovery	0.5	" " 7 min.
4	"	s.c.	30	9250 "	Immediate symptoms. Death in 72 min.*		
5	"	"	30	12050 "	No symptoms	0.04	" " 5 min.
6	"	"	30	23640 "	Severe typical symptoms with recovery	0.5	Severe symptoms with recovery
7	"	"	30	9800 "	No symptoms	0.04	Severe symptoms with recovery
8	"	"	30	11450 i.v.	" "	0.1	No symptoms
9	"	i.p.	30	8850 i.c.	Mild "	0.04	" "
10	"	"	30	9100 "	No "	0.02	" "
11	"	"	30	8100 "	Mild "	0.04	Moderate symptoms

* Intrapleural hemorrhage at necropsy.

and other symptoms in 5.7% of 209 cases which he considers an atypical sensitization phenomenon because of the transient character of the period of sensitivity.

The unexplained nature of the toxic reac-

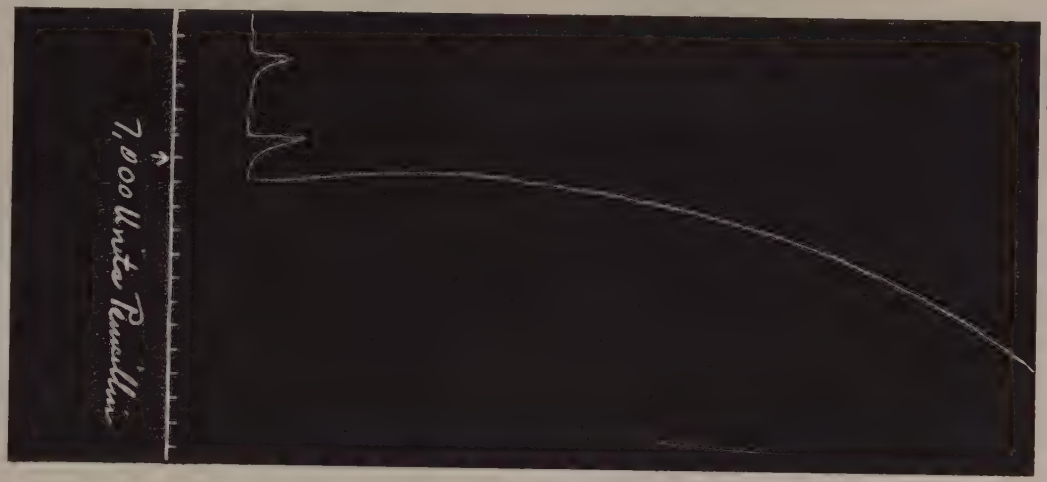

FIG. 1.
Guinea pig sensitized by five intraperitoneal injections of 500 Oxford units each of penicillin given at daily intervals. Test performed after an incubation period of 20 days. Typical response to 7,000 units in 100 cc oxygenated Ringer-Locke solution. Time in 30 seconds.

TABLE II.
Anaphylactic Response of the Isolated Uterus to Penicillin. Both Cornua Used.

No.	Sensitizing dose, units	Incubation period, days	Shocking dose units	Response	Desensitization
1	4 x 500 + 1 x 1000 s.c.	16	(a) 3000	None	
			(b) 7000	"	
2	5 x 500 i.p.	19	(a) 3000	"	
			(b) 7000	Positive delayed	
3	" "	20	(a) 7000	"	Ineffective
			(b) 2000	" delayed	"
4	" "	21	(a) 2000	None*	
			(b) 2000	"	
5	" s.c.	23	(a) 2000	"	
			(b) 2000	"	
6	" "	25	(a) 8000	" *	
			(b) 15000	" *	
7†	" i.p.	29	(a) 15000	Positive delayed	"
			(b) 15000	None	
8†	4 x 500 + 1 x 1000 i.p.	31	(a) 15000	"	
			(b) 15000	"	
9†	" "	32	(a) 3000	"	
			(b) 3000	Doubtful	
10†	5 x 500 s.c.	34	(a) 20000	None	
			(b) 20000	Positive delayed	Effective
11†	4 x 500 + 1 x 1000 s.c.	35	(a) 20000	None	
			(b) 20000	"	
12†	5 x 500 s.c.	36	(a) 20000	Positive delayed	"
			(b) 20000	None	
12	1 x 50000 i.v.	13	(a) 20000	"	
			(b) 20000	"	

* Response to standard dose posterior pituitary extract (0.12 mg) subnormal.
† Also sensitized to horse serum with typical response including desensitization.

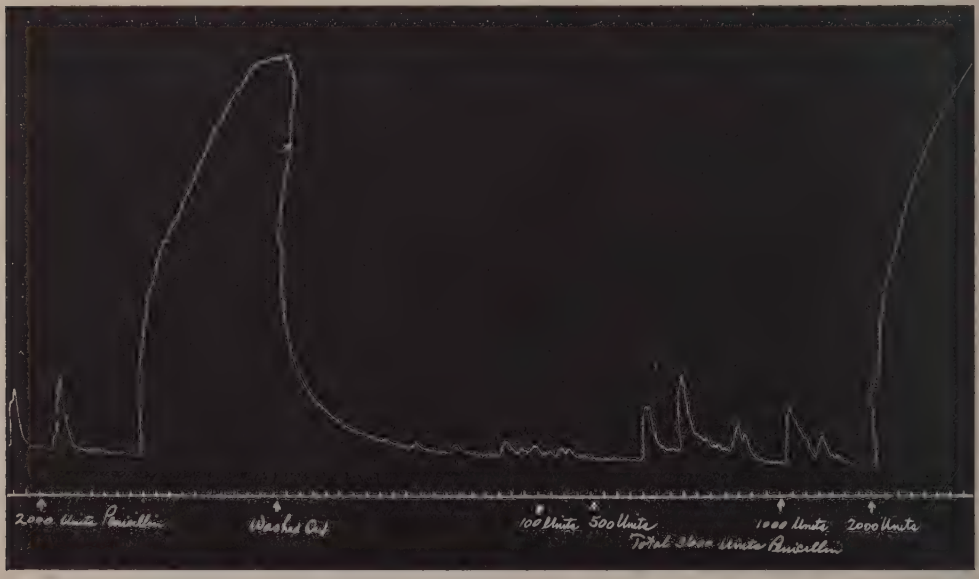

FIG. 2.
Second uterine cornu of same animal as described in Fig. 1. Note delayed reaction, and ineffective desensitization.

tions, though of infrequent occurrence, prompted a study of the substance in guinea pigs to ascertain whether or not it would be possible to demonstrate anaphylactic reactions by the usual methods of sensitization. The primary toxicity of penicillin in guinea pigs was studied by Hamre and associates[5] who showed that the acute fatal dose injected intravenously is upwards of 50,000 units per kg while 7,000 to 12,000 units per kg given daily by the subcutaneous route cause death in several days.

In the present experiments 2 series of guinea pigs were sensitized by daily subcutaneous or intraperitoneal injections of penicillin over a period of 5 days; one group to be used for intracardiac or intravenous injection of the antigen after an adequate incubation period, and the other for tests on the isolated uterus by the Schultz-Dale technique. The individual sensitizing doses ranged from 300 to 1,000 Oxford units and the shocking doses ranged from 5,000 to 23,000 units in the intracardiac group and from 2,000 to 20,000 units in the isolated uterus group. Some of the guinea pigs in each group were also sensitized to horse serum, the object being to ascertain the extent of anaphylactic state in the animals in the event that the penicillin reactions were negative. The sensitizing dose of horse serum was uniformly 0.1 cc and the magnitude of the shocking dose varied as shown in the tables. In the latter group one animal was sensitized by a single intravenous injection of 50,000 Oxford units.

The results of these tests are shown in two tables. Table I gives a detailed account of 11 animals tested after a suitable incubation period by the intravenous or intracardiac route. Of these, 2 died with typical symptoms of anaphylactic shock and 2 showed moderate to severe symptoms followed by recovery. Five in this group were refractory and 2 showed mild symptoms of a doubtful nature. It is to be noted, however, that of the 7 animals refractory to penicillin 3 were also wholly refractory to horse serum and 2 though responding with typical symptoms to the horse serum made good recoveries. The shocking dose of horse serum was injected in the same manner as the shocking dose of penicillin.

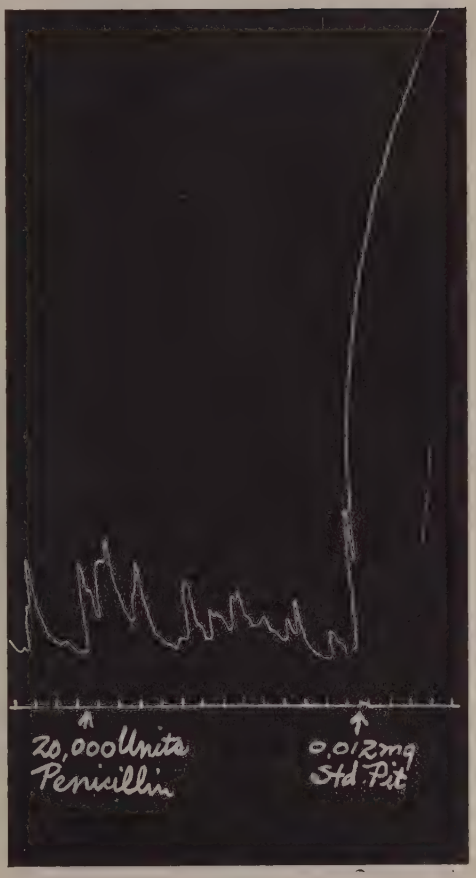

FIG. 3.

Shows negative response to 20,000 units in a guinea pig sensitized by 4 daily doses of 500 units and 1 of 1,000 units injected subcutaneously, and tested after an incubation period of 35 days. The response to a test dose of pituitary was normal.

In Table II are given the results with the isolated uterus method. In this there were 13 animals, each uterine cornu having been subjected to the test, making a total of 26 tests. A positive response was elicited in 6 preparations obtained from 5 animals. The preparations of 7 animals gave negative results, and one doubtful. The physiological condition of the uterine horn was tested in each case by a small dose of posterior pituitary extract, and

[5] Hamre, D. M., Rake, G., McKee, C. M., and MacPhillamy, H. B., *Am. J. Med. Sci.*, 1943, **206**, 642.

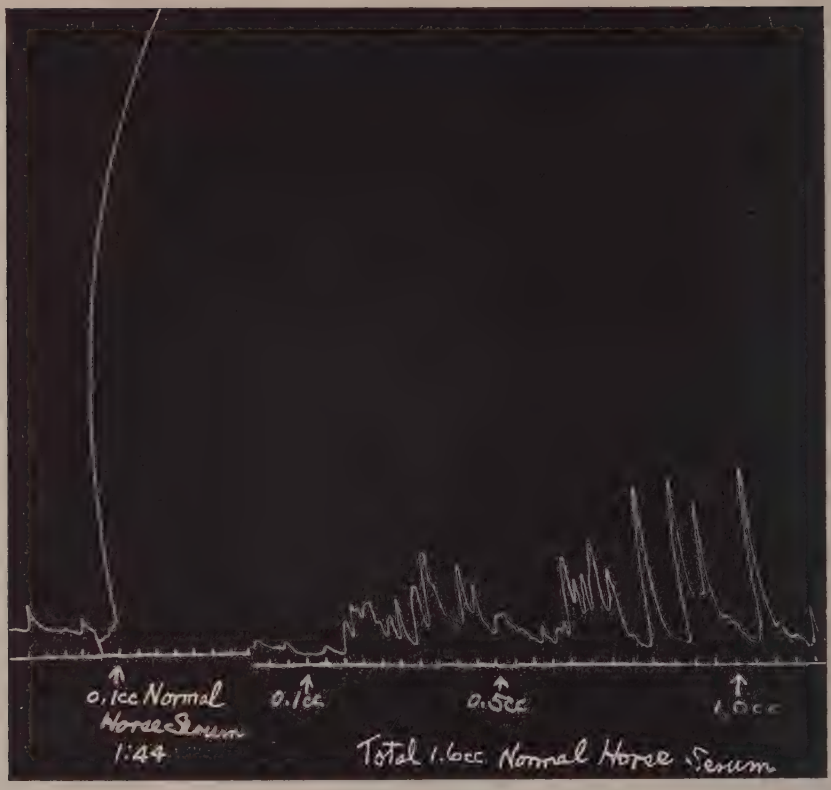

FIG. 4.
Same preparation as shown in Fig. 3. Note typical response to horse serum and characteristic desensitization.

in 6 of the experiments which had been sensitized to horse serum as well a typical response was produced by the latter antigen. In those experiments in which a positive response was obtained to penicillin desensitization was attempted but it frequently was ineffective. Moreover, the penicillin response when present was delayed, all of which indicates that while sensitization to penicillin is possible it is not uniform and when present is atypical.

In a series of 8 normal guinea pigs under the same experimental conditions penicillin had no appreciable effect on the contractions of the isolated uterus in doses of from 20,000 to 30,000 units. Each uterine horn was tested separately, making a total of 16 tests.

Comment. The present experiments indicate that anaphylactic sensitization in the susceptible guinea pig can be achieved with the penicillin as currently prepared and marketed. The penicillin used in this work consisted of several commercial lots of the sodium salt manufactured by at least 5 different distributors, and was of the same degree of purity as that used currently in the clinic. To what extent impurities alone may have been responsible for some of our results cannot be stated at present. If the reactions observed were due to impurities it is obvious that they are inherent in the commercial products in which they are inseparable from the active substance. Parallel experiments with the purest obtainable crystalline material, or preferably synthetic penicillin, would determine this point. In any event, this work appears to afford at least a partial explanation for some of the untoward effects reported in the clinical use of the drug as supplied commercially. It should also be

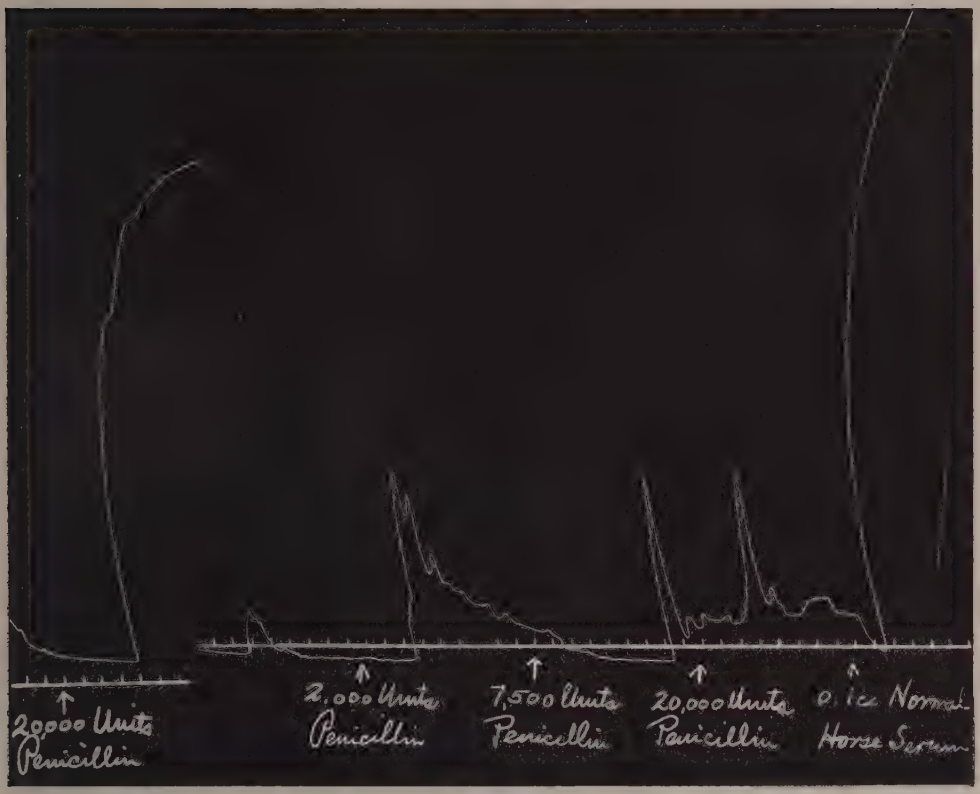

FIG. 5.

Guinea pig sensitized to penicillin and horse serum. Tested for sensitization against both antigens after an incubation period of 36 days. Note prompt response to horse serum and positive though delayed reaction to penicillin with effective desensitization.

pointed out that the positive reactions are for the most part atypical, in that the reactions are often delayed, and desensitization often ineffective. It would seem that the penicillin (commercial) antibody-antigen combination lacks permanency and is more readily reversible than is the case in anaphylactic reactions following sensitization with true proteins. The incomplete and imperfect sensitization with penicillin may perhaps be explained on the supposition that the precipitin-antigen union in this case results in too rapid flocculation to effect cell permeability (Dale[6]). The imperfect antibody-antigen union would also have the effect of failing to saturate or exhaust anaphylactic antibodies, in this case the smooth muscle receptors, which according to Weil and Coca[7] is an essential factor in the mechanism of desensitization.

Summary and Conclusions. Tests for anaphylactic sensitization with penicillin were attempted by the isolated uterus technique and by intracardiac or intravenous reinjection of antigen. Sensitization with penicillin as currently prepared and marketed was demonstrated by both methods. Sensitization was not uniform, and usually atypical in that the uterine response was often delayed and desensitization often difficult to demonstrate. The results appear to afford a partial explanation for some of the reactions encountered in the clinical use of the drug.

[6] Dale, H. H., *Bull. Johns Hopkins Hospital*, 1920, **31**, 310.

[7] Weil, R., and Coca, A. F., *Z. Imm.*, 1913, **17**, 141.

14781

Local Anesthetic Activity of a Series of Phenyl Piperidine Derivatives.*

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From the Department of Pharmacology, Temple University School of Medicine, Philadelphia, Pa.

A number of piperidine derivatives (Table I) have been made available† to us for pharmacological study. The structure of these compounds suggested the present experiments which were designed to determine if they exhibited local anesthetic properties. Comparisons of their activity were made with the diethylaminoethyl ester of aminobenzoic acid (procaine) and with methyl benzoyl ecgonine (cocaine).

Dermal Anesthesia. The technic described by Rose[1] has been employed to detect the presence or absence of anesthesia after intradermal injection of the piperidine compounds in guinea-pigs. The standard of comparison for duration of anesthesia was procaine. In the present experiments, 1.0% solution of procaine produced anesthesia for an average period of 28 minutes, and the piperidine compounds for average periods of from 41 to 75 minutes (Table I). While the anesthesia produced by the piperidine derivatives was more prolonged than that obtained after procaine, all of the new derivatives caused hyperemia, followed in most cases by necrosis at the site of injection.

Corneal Anesthesia. The presence or absence of corneal anesthesia was tested in rabbits using the method previously described.[2] One per cent solution of cocaine hydrochloride, which served as the standard of comparison for duration of topical action, produced anesthesia for an average of 21 minutes (Table I). One per cent solutions of III, IV, V, VII, VIII and IX produced anesthesia for periods of from 21 to 90 minutes (Table I). All of these caused irritation as evidenced by capillary engorgement, except III, which in 1.0% solution did not cause pitting or appear irritating. Compounds VI, X, XI and XII in 1.0% solution failed to induce anesthesia of rabbit cornea by topical application.

Toxicity. In mice by intraperitoneal injection the MLD of compounds III, IV, XI and XII appeared to be in the neighborhood of 100 mg/kg (Table I). The MLD of VIII and IX is approximately 150 mg/kg of body weight and that of V above 100 (Table I). In these experiments the MLD of cocaine hydrochloride was found to be between 100 and 150 mg/kg of body weight (Table I).

Summary. A number of new phenylpiperidine derivatives (α-4-methyl-(III), β-4-methyl-(IV), 4-dimethyl-(V), 4-methylol-(VI), α-4-phenyl-(VII), β-4-phenyl-(VIII), α-4-carboxylic acid-(X), α-4-carbomethoxy-(XI), α-4-carbethoxy-(XII), 3-phenyl-piperidine) were found to produce anesthesia after intradermal injection in guinea-pigs which was more prolonged than that observed after procaine. All of the new compounds induced hyperemia followed in most cases by necrosis at the site of injection. After topical application 1.0% solutions of III, IV, V, VII or VIII produced corneal anesthesia in rabbits. In 1.0% solutions VI, X, XI or XII failed to exhibit topical activity in rabbits. Intraperitoneally in mice the acute toxicity of the new derivatives was in the range of that of cocaine.

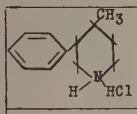
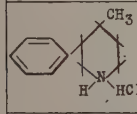
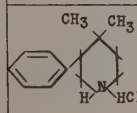
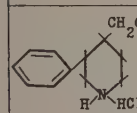
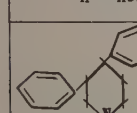
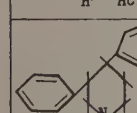
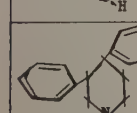
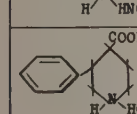
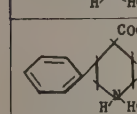
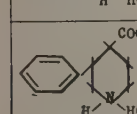
* This investigation was supported in part by the Smith, Kline & French Fellowship Fund.

† These compounds were prepared and made available to us by Dr. Frederick Koelsch of the Department of Organic Chemistry, University of Minnesota.

1 Rose, C. L., *J. Lab. Clin. Med.*, 1929, **15**, 128.

2 Fellows, E. J., Proc. Soc. Exp. Biol. and Med., 1943, **53**, 7.

TABLE I.
Duration of Local Anesthesia and Toxicity.

Formula	Code No.	M.P. °C	Duration of anesthesia: Guinea pig skin 1% min.	Duration of anesthesia: Rabbit cornea 1% min.	Toxicity Mice—Intraperitoneally No. died/No. used mg per kg: 100	Toxicity Mice—Intraperitoneally No. died/No. used mg per kg: 150
	Cocaine			21	5/10	10/10
	Procaine		28			
	III	170-175	58	26	7/8	
	IV	248-252	58	21	8/8	
	V	274-276	49	34	5/8	
	VI	105-111*	41	No anesthesia	Not tested	
	VII	194-198	75	50	4/11	
	VIII	115-116	69	40	3/20	8/8
	IX	160-162 H_2O off at 140	63	90	2/20	8/8
	X	>275	49	No anesthesia	Not tested	
	XI	253-255	73	" "	7/8	
	XII	245-246	56	" "	6/6	

* B.P. 215-218°.

14782P

Renal and Hepatic Injury in Trypsin "Shock".*

I. Arthur Mirsky and Edward D. Freis.

From the AAF Regional Station Hospital, Lincoln Army Air Field, Lincoln 1, Nebraska.

Among the various features of the shock syndrome which develops in consequence of extensive tissue damage (burns, traumatic injuries, abdominal emergencies, severe infections, anoxia) are the negative nitrogen balance and the characteristic renal and hepatic changes. These, and other aspects of the shock syndrome have been attributed to or associated with a variety of "poisons" such as histamine, H-like substances, adenyl compounds, abnormal globulins, vasoconstrictor substances and numerous other products.

It has been demonstrated that the administration of proteolytic enzymes can induce the liberation of a variety of catabolic products, such as histamine[1] "slow reacting muscle stimulant substance" and adenyl compounds[2] and other pharmacologically active substances. Consequently, it occurred to us that with extensive tissue damage a proteolytic enzyme may be released locally, and it in turn be responsible not only for the production of toxic substances but also for an increased proteolysis and the consequent development of a negative nitrogen balance such as is observed in practically all types of shock. In order to test this hypothesis we studied the effects of injections of a known proteolytic agent.

The present preliminary report is concerned only with the renal and hepatic changes observed in rats and rabbits which received injections of a crude preparation of trypsin.† The intraperitoneal injection of a sterile 2% to 4% solution of trypsin in saline resulted in the development of the shock-like syndrome in from 3 to 36 hours, when from 5 to 10 cc was administered to rats and 10 to 20 cc to rabbits. A simlar syndrome could be induced in rabbits by the repeated intravenous injection of a dilute solution of trypsin. Thus, when young rabbits were injected 8 times daily for 2 days with 10 to 20 cc of a 0.25% trypsin solution, collapse and death occurred in from 24 to 36 hours after the last injection.

Postmortem examination of rats which died with the shock-like syndrome revealed some degree of peritonitis in some animals and not in others. The majority of 16 rats thus studied showed a marked congestion of the liver, soft and somewhat swollen kidneys and usually, a pale contracted spleen. A similar picture was observed in the rabbits which died, though signs of peritoneal irritation were not found.

Microscopic examination of the kidneys of both rats and rabbits revealed various degrees of damage from cloudy swelling and vascular congestion to degeneration of the cells of the collecting and distal convoluted tubules, massive deposition of eosinophilic granular and fibrillar material in the tubules and glomerular spaces, and focal collections of lymphocytes in the interstitial tissue about the degenerated distal convoluted tubules and arterioles. In some tubular spaces the contained material formed rounded hyaline bodies resembling red blood cells. The similarity to the pathologic changes observed in the kidneys of man dying from "crush injuries"[5] or burns is striking.

The liver showed various degrees of damage from cloudy swelling and vascular congestion to focal or massive necrosis and infiltration of the portal areas with neutrophiles and lymphocytes.

* AAF Rheumatic Fever Control Program.

1 Roche e Silva, M., *Arch. Exp. Path. u. Pharmak.*, 1940, **194**, 335.

2 Trethewie, E. R., *Austr. J. Exp. Biol. Med. Sci.*, 1942, **20**, 49.

† Fairchild's Trypsin and Wilson's Trypsin were employed. We are indebted to Dr. David Klein of the Wilson Company, Chicago, Ill., for generous supplies of a potent trypsin preparation.

In addition to the morphologic data herein reported, we have observed that the urine from animals in shock frequently contained large quantities of R. B. C., hyaline, and granular casts. Conclusive evidence of an hemoglobinuria was not obtained.

It must be emphasized that the intravenous injection of concentrated trypsin solutions (2% to 4%) in rabbits results in rapid death, within a few minutes after the injection, or even while the injection is in progress. Examination of such animals reveals extensive intravascular clotting, all chambers of the heart being filled with thrombus. In some respects this response is similar to the anaphylaxis-like syndrome produced by trypsin as described by Roche e Silva[3] and Dragstedt[4] although they do not attribute the collapse and death to the intravascular clotting which we have observed.

Data not yet reported lend support to the possibility that with extensive tissue damage some proteolytic enzyme is released or activated which in turn may be responsible not only for changes at the site of injury, but also for the production of catabolic factors whose effects may be reflected in renal, hepatic, and other systemic functional and structural depressions.

3 Roche e Silva, M., *Arquivos d. Inst. Biol.*, 1939, **10**, 93.

4 Dragstedt, C. A., and Wells, J. A., *Quart. Bull. Northwestern Univ. Med. Sch.*, 1944, **18**, 104.

5 Bywaters, E. G. L., and Beall, D., *Brit. Med. J.*, 1941, **1**, 427.

14783P

Paroxysmal Pulmonary Edema: A New Experimental Method.

ALDO A. LUISADA AND STANLEY J. SARNOFF. (Introduced by M. D. Altschule.)

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We have studied pulmonary edema following rapid infusion of massive doses of fluid into the circulation of dogs. Infusion into either the femoral or the jugular veins, or the femoral arteries was tried in 14 dogs with inconstant results. The rapid infusion of fluid into the carotid arteries toward the brain was therefore used to induce pulmonary edema constantly.

Technique. Experiments were performed on 18 dogs weighing from 8 to 20 kg, under morphine (3 mg/kilo)-urethane (1 g/kilo) anesthesia. Infusions were given simultaneously into both carotid arteries under a pressure of 280-300 m/m Hg. The total quantity of fluid injected amounted to 2.3 times the animal's estimated blood volume (on the basis of an assumed blood volume equal to 10% of body weight). A first infusion amounted to 85% of the blood volume and required from 1 to 2.5 minutes. A second infusion equivalent to 80% was given 10 minutes later. A third infusion equivalent to 65% of the blood volume was given 5 minutes after the end of the second. If the animal survived, it was sacrificed 7 minutes after the end of the last infusion.

Edema of the lungs was evaluated by: (a) weighing the lungs and calculating the lung/body index; (b) examining the gross appearance of the lungs and the presence of foam in the trachea; (c) studying the fluid pouring out of the cut parenchyma. Venous pressure, arterial pressure, X-ray of the chest and electro- and phonocardiograms were studied in some animals.

Results. 16 of the group of 18 dogs studied developed severe pulmonary edema after infusion with: (a) Tyrode solution; (b) physiologic salt solution; (c) bovine albumin solution; (d) oxygenated dog's blood.

Pulmonary edema occurred in all 4 types of infusion, with increasing severity from (a) to (d), but dyspnea was more severe with saline than with the other fluids.

Having obtained a reliable technique, we

have studied the effect of different surgical and mechanical procedures, and drugs, on the occurrence and severity of the edema. These studies are continuing and, therefore, only a brief summary of the results is given here.

Procedures useful in preventing or minimizing the edema were: (a) Intratracheal positive pressure; (b) bleeding during the infusion, and (c) denervation of the carotid sinus.

Useful drugs were: (a) Drugs inhibiting the sympathetic system (883 Fourneau,* ergotamine); (b) Curare; (c) Narcotics (barbital, phenobarbital, morphine, chloral); (d) Procaine (subcutaneous injections); (e) A combination of atropine and physostigmine.

Comment. A definitive interpretation of the results will be possible only after completion of the study. However, the experiments appear to show that stimulation of the cardiovascular receptors (chiefly those of the carotid sinus) has a prominent part in the genesis of our type of experimental pulmonary edema by increasing in a reflex way the permeability of the lung capillaries.

* 2-diethylamin-ethyl-1,4-benzodioxan is the formula of 883F.

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Experimental Alloxan Diabetes in Hooded Rats.

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Following the demonstration of Shaw Dunn and his collaborators[1,2] that alloxan, administred intravenously, produces rapid necrosis of the islets of Langerhans in rabbits, various investigators have observed the same effects on the islets and the development of persistent diabetes following parenteral injections of alloxan in rabbits,[3-6] white rats,[7, 8] and dogs.[9, 10] Gomori and Goldner[8] failed to produce these effects in 5 hooded rats given intraperitoneal injections of alloxan in doses of 200 mg per kg of body weight and, accordingly, they considered hooded rats resistant to alloxan.

On the contrary, we have found islet lesions and disturbances of blood sugar levels without exception in more than 60 hooded rats injected subcutaneously with alloxan (Eastman) in doses of 175 to 350 mg per kg of body weight. In these experiments a single injection of a 5% solution of alloxan was employed in each case. Blood sugar determinations were made by a modified Folin micro method as frequently as every 1 or 2 hours during the first 12 hours, every 3 to 6 hours up to 48 hours and at longer intervals thereafter. The animals either died, were killed when moribund or were sacrificed at desired intervals. Complete autopsies were performed in every instance and tissues appropriately fixed for histological staining with haematoxylin and eosin, Best's carmine stain for glycogen and Gomori's stain for granules in islet cells of the pancreas.

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3 Bailey, C. C., and Bailey, O. T., *J. A. M. A.*, 1943, **122**, 1165.

4 Hughes, H., Ware, L. L., and Young, F. G., *Lancet*, 1944, **1**, 148.

5 Goldner, M. G., and Gomori, G., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 73.

6 Hard, W. L., and Carr, C. J., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 214.

7 Dunn, J. S., and McLetchie, N. G. B., *Lancet*, 1943, **2**, 384.

8 Gomori, G., and Goldner, M. G., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 287.

9 Goldner, M. G., and Gomori, G., *Endocrinology*, 1943, **33**, 297.

10 Brunschwig, A., and Allen, J. G., *Cancer Research*, 1944, **4**, 45.

Within one hour after the injection of alloxan the blood sugar level had begun to rise from its normal average of about 100 mg % and at two hours reached a peak of from 130 to 240 mg %. Therefter the blood sugar fell to normal or below normal by the end of 4 to 6 hours and rose again above the normal level within 6 to 24 hours. Hypoglycemia did not always occur; the lowest blood sugar level recorded during this phase was 52 mg %. Hypoglycemic convulsions were not observed. Urine collected during the first 6 hours regularly showed a pink color, not due to haematuria or haemoglobinuria, but resembling closely the color that results when alloxan solution is allowed to evaporate in air. Glycosuria was present within the first 24 hours in all cases of marked hyperglycemia.

After the first 24 hours the results varied with the dosage of alloxan and apparently also with the individual susceptibility of the animals. Usually with the larger doses of alloxan (300 to 350 mg per kg) and sometimes with smaller doses, the blood sugar rose rapidly within 30 to 48 hours to values of 800 to 1450 mg % and death occurred within 36 to 96 hours in a state suggestive of diabetic coma accompanied by massive glycosuria and acetonuria. On the other hand, with smaller doses (175-200 mg per kg) and sometimes with larger doses, the blood sugar rose gradually within 2½ to 5 days to a peak of about 500 mg %. The animals survived for weeks or even months without treatment, with blood sugar levels of 300 to 500 mg%. These rats exhibited marked glycosuria (excreting as much as 5.5 g of sugar per day) as well as polyuria, polydypsia, polyphagia and progressive loss of weight when maintained on their ordinary diet of Purina Fox Chow without carbohydrate supplement.

The earliest histological examination of tissues was made one hour after injection of 350 mg per kg of alloxan and alterations were already discernible in some of the larger islets of Langerhans in the form of moderate nuclear pyknosis of a few of the central cells. During the next few hours, pyknosis of nuclei became more marked and extensive. The affected cells appeared markedly distended owing to great swelling of the cytoplasm; the cells became detached from one another and their normal cord-like arrangement was distorted. This stage was followed by fragmentation of nuclei and fading of nuclear staining with rupture of the cell membranes. By the end of 24 hours, as a result of complete disintegration of cells, the centres of many of the islets were occupied only by pale staining granular debris partly surrounded by clusters of surviving peripheral cells. The picture of more or less complete and widespread dissolution of islets was most conspicuous between 24 and 72 hours. Within the next 2 or 3 days there was a gradual condensation and disappearance of the necrotic debris with a corresponding collapse and shrinkage of the affected islets. Finally, although the general impression was one of paucity of islets in the histological sections, careful search revealed the presence of small solid clusters of surviving islet cells. The changes described were less extensive and developed more slowly with smaller doses of alloxan.

While the majority of the islet cells destroyed were beta cells, some of the more centrally placed alpha cells were also observed to be necrotic. The majority of the peripheral alpha cells evidently survived the injury and formed the greater part of the small islet cell clusters that remained to represent the original islets. In addition, however, some of the shrunken islets contained a few cells with clear agranular pale staining cytoplasm.

Although all previous investigators who have commented on the state of the acinar tissue of the pancreas following injections of alloxan have reported the absence of significant alterations, we have found in the first 24 hours after injection a striking increase in mitotic activity in the acinar cells. In sections of the pancreas from control animals, the most careful search revealed only rare mitotic figures in acinar cells, but beginning at 2 hours after injection of alloxan the number of mitotic figures was significantly increased. This increase continued, reaching a maximum at 17 hours when as many as 287

mitotic figures were counted in one section containing several small fragments of pancreatic tissue. Thereafter, the number of mitoses decreased to approximately normal rarity by the end of 24 hours. The distribution of acinar cells in mitosis appeared to be completely haphazard. The even smaller numbers of mitotic figures observed in the pancreatic ductular epithelium of control animals were not discernibly increased in the experimental animals. No mitotic figures were seen among islet cells at any time.

With the exception of slight degenerative changes in the convoluted tubules of the kidneys and the irregular occurrence of slight fatty metamorphosis of the liver, no significant alterations were observed in organs other than the pancreas, including the other endocrine organs. Histological alterations of the adrenal medulla similar to those described by Hard and Carr[6] were encountered irregularly in both experimental and control animals.

Sections of the liver from diabetic animals were found histologically to be lacking in glycogen. The heart muscle contained some stainable glycogen but this was not distinctly greater in quantity than normal. The deposition of glycogen in the renal tubular epithelium, especially of the loops of Henle, was satisfactorily demonstrated in several diabetic animals.

Summary. Alloxan administered to hooded rats in single subcutaneous injection in doses of 175 to 350 mg per kg of body weight regularly produced rapid selective necrosis of the islets of Langerhans and characteristic fluctuations of the blood sugar level. During the first 24 hours, irrespective of dosage, a blood sugar curve of typical form was obtained. Thereafter, following the higher doses of alloxan, the blood sugar rose rapidly to very high values and death occurred in a state resembling diabetic coma. With the lower doses employed the animals survived for periods up to several months with persistent diabetes. Histological alterations in the islets of Langerhans conformed to those described by previous investigators in white rats following alloxan. In addition, a great increase was observed in the activity of mitotic division in acinar cells of the pancreas during the first 24 hours after injection of alloxan.

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Transmission of Penicillin Through Human Placenta.*

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This study was undertaken to determine the existence of a placental barrier to penicillin.

Experimental. Four normal patients were selected while in active labor. Delivery was expected within 2 hours. Penicillin,† dissolved in normal saline, was administered intravenously. Ten cubic centimeters of saline were used irrespective of the dose administered. The calcium salt of penicillin was used in one case, the sodium salt in the remainder.

Samples of the maternal blood were taken for assay at intervals from the time of injection up to and including the time of delivery. At the time that the baby was separated from the placenta, placental cord blood was withdrawn into a sterile test tube. In one instance (Case 1) a pipette was inserted into the uterus following spontaneous expression of the placenta. Blood was aspirated from the uterus for assay. In Case 3 the amniotic sac was perforated about 5 minutes prior to delivery and amniotic fluid obtained for assay.

* This manuscript was submitted for publication June 1, 1944.

† The penicillin used throughout this study was supplied through the kindness of Mr. John L. Smith, Chas. Pfizer & Co., Brooklyn, N. Y.

TABLE I.
Amount of Penicillin in Maternal Blood at Intervals Following Intravenous Administration.

Cases	Dosage (units)	Maternal blood			
		½ hr	1 hr	1½ hr	2 hr
		Units per cc			
1 H.B.	100,000	0.32	0.16	—	0.04
2 M.P.M.	100,000	2.56	0.32	0.16	0.01

TABLE II.
Amount of Penicillin in Maternal and Placental Cord Blood at Time of Delivery.

Cases	Dosage (units)	Time* (hr)	Maternal blood (units/cc)	Placental cord blood (units/cc)	Amniotic fluid
1 H.B.	100,000 Ca	2	0.04	0.02	
2 M.P.M.	100,000 Na	2	0.01	0.02	
3 L.C.	20,000 Na	½	0.19	0.05	Neg.
4 A.B.	20,000 Na	1	0.094	<0.047	

* Time is period elapsed from the administration of penicillin to delivery of infant.

In each case blood was allowed to clot and was then stored over-night at refrigerator temperature. On the following day the serum was withdrawn using sterile precautions.

Serum was tested for the presence of penicillin by the method of Dawson, Hobby, *et al.*[1,2] Hemolytic streptococcus (strain C203Mv) was used throughout. The serum was diluted serially from 1:2 to 1:512 in a phosphate buffered beef infusion medium. Hemolytic streptococci were added in an amount sufficient to give a final dilution of 10^{-6}. Incubation was carried out at 37°C for 24 to 30 hours. Standard penicillin of known unitage, diluted serially from 3 to 0.0012 Units per cc, was run simultaneously.

In 2 cases, determinations of the amount of penicillin in the maternal blood were made at one-half hour intervals following injection in order to ascertain rapidity of elimination from the blood stream in the woman in labor. As shown in Table I, penicillin was eliminated at approximately the same rate as was previously demonstrated in other patients by Rammelkamp & Keefer[3] and by Dawson *et al.* [1]

It is evident from Table II that penicillin surmounts the placental barrier. When one hundred thousand units were used, the concentration in cord blood was relatively high even though 2 hours had elapsed following administration of the penicillin. When twenty thousand units were used the concentration in cord blood one-half hour after administration was adequate to inhibit sensitive bacteria. If the time was extended to one hour, the amount in the cord blood was insignificant. Except for one instance, there was a lower level in the cord blood than in maternal blood. There was no evidence of toxicity to mother or baby.

Summary. Penicillin was administered to 4 normal patients in active labor. Two patients received one hundred thousand units each. Both showed significant amounts of penicillin in the maternal and placental cord blood, 2 hours after administration. Two patients received twenty thousand units of penicillin each. The patient who received it one-half hour before delivery showed ample penicillin in the maternal and placental cord blood but not in the amniotic fluid. The patient who received penicillin one hour before delivery showed significant penicillin in the maternal blood but none in the placental cord blood.

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3 Rammelkamp, C. H., and Keefer, C. S., *J. Clin. Invest.*, 1943, **22**, 425.

14786

Influence of Acute Changes in Blood Pressure on the Distribution of Fibrinogen.*

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Observations on fibrinogen completed in the course of studies on histamine shock previously reported[1] are included in the communication that follows. The acute fall and rise in the fibrinogen level of plasma, as will be seen, does not parallel those of other plasma proteins[2] but conforms to the acute pressure changes. The evidence indicates that the fibrinogen content of the circulating blood is coordinated with the sudden change in size of the smaller vessels.

Material and methods. Dogs anesthetized with 25-30 mg of nembutal per kg of body weight, rarely with morphine—amytal, received intravenously 2-6 mg of histamine dihydrochloride. The blood pressure was recorded from the femoral artery with a mercury manometer. Hematocrit readings were made on 10 ml samples of blood rendered incoagulable by addition of 20 mg of solid sodium oxalate and centrifuged for 25 minutes at 2000 r.p.m. Total plasma proteins were estimated by the Kjeldahl method. Fibrinogen values were determined in duplicate as fibrin by the Cullen-Van Slyke method[3] and as fibrinogen by the protamine precipitation method.[4] Both methods were carried out in standardized dilutions of 1 ml of plasma in 25 ml of saline. Albumins and globulins were calculated by deducting the protamine precipitable fibrinogen values from the total plasma protein values.

Changes in the level of fibrinogen in the femoral vein. The averaged results of 28 experiments are compiled in Table I. Concomitant with, but lagging somewhat behind the falling blood pressure a marked absolute as well as a relative decrease of fibrinogen occurs. The maximal loss per unit of plasma is reached in about 3-5 minutes after the administration of the drug and amounts to an average of 49.2% for fibrin and 30.1% for protamine precipitable fibrinogen. With slight delay as compared to the climbing arterial pressure the fibrinogen tends to return toward the preinjection level. But like the pressure it usually does not quite reach this height. During the same interval the plasma albumins and globulins show only minor change. In column 6 of Table I the fibrin values are expressed in relation to the albumin and globulin values. This index shows a 48.1% decline at the peak of the fibrinogen loss. When the plasma fibrinogen level has reached a postinjection maximum at around 30 minutes, there is a secondary progressive decline that is paralleled by a similar fall in albumins and globulins. Reinjection of histamine, on the other hand, is followed by the same marked loss in fibrinogen as compared to other plasma proteins.

Columns 3 and 4 of Table I show the fibrinogen values as estimated by the two methods to follow the same trend. Quantitatively the protamine precipitation method yields higher absolute values than the fibrin method. The difference reaches a maximum with the peak of the fibrinogen loss, to be reduced again toward the end of the experiment. The loss in fibrinogen is consequently more pronounced by the fibrin method.

* Aided by grants from the John and Mary R. Markle Foundation.

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4 Mylon, E., Winternitz, M. C., and deSütö-Nagy, G. J., *J. Biol. Chem.*, 1942, **143**, 21.

5 Jones, T. B., and Smith, H. P., *Am. J. Physiol.*, 1930, **94**, 144.

TABLE I.
Changes in Plasma Protein Constituents of Dog After Intravascular Administration of Histamine. The figures are computed from 28 identically arranged experiments.

Time, min	Mean arterial blood pressure, mm Hg	Fibrinogen as fibrin, %	Fibrinogen as protamine precipitate, %	Albumins and globulins, %	Fibrinogen as fibrin × 100 / Albumins and globulins, %
0	136	0.459	0.485	4.96	9.25
	Intravascular administration of 2-6 mg of histamine dihydrochloride.				
1½-2	40	0.303	0.377	4.90	6.18
4-5	45	0.233	0.339	4.84	4.81
10	87	0.307	0.362	4.83	6.85
30	102	0.378	0.401	5.00	7.56
60	103	0.338	0.365	4.70	7.19

TABLE II.
Effect of Epinephrine on the Blood Composition of the Dog.

Time, min	Mean arterial blood pressure, mm Hg	Fibrinogen as fibrin, %	Fibrinogen as protamine precipitate, %	Albumins and globulins, %	Hematocrit, %
0	105	0.158	0.172	4.58	51.5
	Intravascular administration of 0.5 ml of 1:10,000 epinephrine solution.				
3	165	0.278	0.245	3.87	42.7
6	105	0.236	0.222	4.45	51.5

Changes in fibrinogen content of blood from different vessels and under different experimental conditions.

Fibrinogen, albumin-globulin and hematocrit estimations were made from samples of blood obtained simultaneously from femoral vein and aorta, carotid and femoral arteries, inferior vena cava, jugular and portal veins. The fibrinogen changes are similar in the blood from all these vessels.‡

The characteristic fall and rise of fibrinogen after histamine injection is uninfluenced by anesthesia and splenectomy.

After cord transsection usually there is a trend towards hemodilution. This trend is temporarily interrupted by histamine. Concomitant with the ensuing hemoconcentration an increase in plasma albumins and globulins actually may occur. In spite of this the fibrinogen decreases with the pressure fall.

The effect of other vasodepressors and a vasopressor on the fibrinogen level. The changes described above for fibrinogen are not specific for histamine. Similar changes follow when vasodepression occurs from other cause, like intravascular injection of peptone,[5, 6] thromboplastic substance,[1] acetylcholine,[7] acute hemorrhage.[6]

With vasopressor substance, like epinephrine, the resultant rise in pressure is accompanied by elevation of plasma fibrinogen[7, 8] that disappears with the return of the pressure to normal. As is seen in Table II the peak of the rise in arterial pressure is reached in about 3 minutes after the injection of 0.5 ml of a 1:10,000 solution of epinephrine. This is associated with marked rise in fibrinogen, amounting to 76% and 42.4%, respectively. A 23% hemodilution, on the other hand, is followed by a 15.5% decline of the plasma albumin and globulin level. The fibrinogen trend as estimated by the two methods is similar but the difference between corresponding values is not constant. The increase in fibrinogen is more pronounced by the fibrin method. The fibrin value in the preinjection sample is 8.7% lower, in the 3 minutes sample 13.5% higher than the protamine precipitable fibrinogen value. At the termination of the experiment it is still higher

‡ Consecutive preinjection samples from different vessels show variations in fibrinogen content of blood.

[6] Unpublished observations.

[7] Goreczky, L., and Berencsi, G., *Z. f. exp. Med.*, 1939, **106**, 495.

[8] Cannon, W. B., *The Wisdom of the Body*, W. W. Norton, New York, 1932.

but the difference has diminished. On the return of fibrinogen to preinjection level the usual relationship is restored.

Summary. The fibrinogen level of plasma and blood of large veins and arteries declines sharply and then gradually returns toward the preinjection level immediately after intravascular injection of histamine. The quantitative changes in fibrinogen are independent of those in other plasma proteins. They parallel the acute drop and gradual return in blood pressure referable to peripheral capillary alterations. This movement of fibrinogen is independent of anesthesia, splenectomy and cord transsection. It is not specific for histamine but occurs with other vasodepressors as well. With vasopressor agents a reversed movement of fibrinogen follows. The sudden changes in size of the capillary bed seem to be coordinated with the fibrinogen content of the circulating blood.

The two methods used for the estimation of fibrinogen yielded qualitatively similar but quantitatively differential values.

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Prophylactic and Curative Effects of Certain Sulfonamide Compounds on Exoerythrocytic Stages in *Plasmodium Gallinaceum* Malaria.

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The discovery by Raffaele[1] of a hitherto unrecognized form in *Plasmodium relictum* malarial infections has aroused considerable interest. They are non-pigmented and develop outside the red cell. They have been seen in *Plasmodium gallinaceum* infections by James and Tate[2] who called them exoerythrocytic schizonts. More recently these forms have been recognized in several other avian malarial infections, but with doubtful exceptions they have not been encountered in monkey or human malaria. A complete summary of recent literature has been compiled by Porter and Huff.[3]

The chief interest in these forms from the standpoint of chemo-therapy has been their refractoriness to quinine or atebrin. It is possible to obtain a marked therapeutic effect on the circulating *Plasmodium gallinaceum* parasites in young chicks by administering quinine or atebrin, yet death usually ensues, and at autopsy overwhelming numbers of exoerythrocytic forms can be found. For this reason it has been postulated by some that they are responsible for initiating relapses since they are not destroyed by the accepted antimalarial drugs. According to Kikuth and Mudrow[4] plasmochin possesses slight activity on the exoerythrocytic stages but no other drug has been shown to affect them.

Since these stages are found rarely in the circulating blood it has been difficult to study their development and to determine their response to chemo-therapy. This handicap has been overcome by perfecting a simple biopsy technic which permits multiple examination of brain tissue in individual animals and thus enables one to follow the course of development in treated and control chicks. The purpose of this preliminary report is to describe the methods used and to show that

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1 Raffaele, G., *Riv. di Malariol.*, 1936, **15**, 318.

2 James, S. P., and Tate, P., *Nature*, 1937, **139**, 545.

3 Porter, R. J., and Huff, C. G., *Am. J. Trop. Med.*, 1940, **20**, 869.

4 Kikuth, W., and Mudrow, L., *Z. f. Immun.*, 1939, **95**, 285.

TABLE I.
Development of Exoerythrocytic Stages in Quinine Treated and Untreated Chicks with *Plasmodium gallinaceum* Infections.

Age of chicks	No. of chicks	Dosage trophozoites	Route	Day of appearance exostages	No. deaths	No. recovered	Day of disappearance exostages
30 days	4	10,000	I.C.	20-25	2	2	35, 39
15	9 Q	100,000	I.V.	19-24	7	2	
13	8 Q	10,000	I.V.	17-20	6	2	26
20	6 Q	10,000	I.V.	17-21	6	0	
15	3 Q	1,000	I.V.	18-22	3	0	
7	2 Q	1,000	I.V.	20-22	2	0	
3	9 Q	200	I.V.	18-21	8	1	
7	7	20,000*	I.V.	8-11	6	1	18
7	12	10,000*	I.V.	8-11	12	0	

Q = Received quinine therapy during acute peripheral blood infection.
I.C. = Intracerebral.
I.V. = Intravenous.
* = Received sporozoites.

certain sulfonamide drugs prevent the appearance of exoerythrocytic forms. Likewise these drugs have a demonstrable curative effect on the forms when given after they have made their appearance in the brain capillaries. Neither quinine nor atebrin retards the appearance or alters the development of the forms. Plasmochin possesses slight activity.

Materials and Methods. White Rock chicks from a single hatchery were routinely used. The parasite was *Plasmodium gallinaceum*.

Infections were induced by injecting viable sporozoites from infected *Aedes aegypti* mosquitoes. Mosquitoes from 3 to 10 days old are allowed to feed upon infected chicks which show approximately 0.4% of the red cells containing gametocytes. It has not been found necessary to demonstrate exflagellation as practically all of the biting females become infected. The infected lots of mosquitoes are stored at 28°C for approximately 15 days, when they are lightly etherized, removed from the cages and rapidly dissected. An operator can remove the salivary glands at an average rate of 90 per hour. The number of infected mosquitoes usually employed to infect a group of chicks may be roughly approximated in the ratio of one mosquito for each chick. The glands are placed in a small vial containing 0.5 cc heparinized normal chick blood. The vial is approximately 2 inches long and contains 3 sections of glass stirring rod each 1½ inches long. The vial is then rolled slowly for 15 minutes and sporozoite counts are made from stained smears of a sample of the mixture. The sporozoites are counted and expressed as the number per 10,000 red cells. An effort is made to get 20,000 sporozoites in each 0.2 cc of the inoculum. If the lots are heavily infected, the number of sporozoites per unit volume can be reduced by dilution or, if scanty, additional infected glands added. The mixture is injected intravenously in 0.2 cc amounts. Parasites usually appear on the sixth or seventh day with death occurring on approximately the fourth day thereafter.

Exoerythrocytic Biopsies. The exoerythrocytic forms are most readily discernible in the brain capillaries. A 0.25 cc syringe containing 0.02 cc of saline fitted with a twenty-guage needle is inserted into the cortex of the cerebrum, and rotated 360°. At the same time slight suction is applied, and upon removal a satisfactory sample of brain tissue can be obtained. This biopsied material is emptied on a glass slide and smeared. Dried and stained with Giemsa stain, it is available for immediate study. Repeated biopsies on the same chick can be made with only occasional damage. Secondary infections are rarely encountered. This simple technic affords an unusual opportunity to observe the development of the exoerythrocytic forms in the living animal.

Development of Exoerythrocytic Forms. In the routine sporozoite-induced infections the exoerythrocytic forms first appear in the

TABLE II.

Effectiveness of Sulfonamides and Quinine on Exoerythrocytic Schizonts in Trophozoite-induced *Plasmodium gallinaceum* Infections. Drugs started on initial appearance of exoerythrocytic forms, one dose per day.

Chick No.		Dosage, mg/os	Days following inoculation										
			16	20	23	24	25	26	27	29	32	35	39
162	Sulfaguanidine	250*	0	+	+			0		0	0	0	0
170	”	250*	0	+	++			+		0	0	0	0
165	Sulfapyridine	500	0	0	+			+		0			0
182	”	500		0	+			0		0	0	0	0
173	Sulfathiazole	500	0	0	+			+d		0	0	0	0
191	”	500		0	+			++		0	0	0	0
169	Sulfadiazine	500			0	0	+	0		0	0	0	0
172	”	500		0	0	+		+		0	0	0	0
179	”	250*	0	++	+++	D							
180	”	250*	0	+	++		+++D						
156	Sulfanilamide	250	0	0	0	0	0	+		0	0	0	0
176	”	500		0	+				+++D				
181	”	500†		0	++					0	0	0	0
153	Quinine	40		0	0	+		++		+	+++	+++	+++
157	”	40	0	+	++	++++D							
161	”	40		++	++++D								
	Controls			0	++			++++D					
				+	+			+		++	+	0	0
				0	0	0	++			++	++	+	0
				+	+++			+++D					

*Dose increased to 500 mg on 23rd day.
†Dose reduced to 250 mg on 29th day.
+ - ++++ = Intensity of exoerythrocytic stages.
d = Damaged.
D = Died.

TABLE III.

Prophylactic Effect of Sulfonamides, Sulfones, Quinine, Atebrin and Plasmochin on Exoerythrocytic Stages following Sporozoite Inoculations. Drug fed in mash from fifth to fifteenth day.

Chick No.	Drug	% of drug in mash	Biopsies on days following inoculations								
			9	10	11	12	13	14	15	16	17
3418	Sulfadiazine	0.5			0			0			0
3419					0			0			0
3420					0			0			0
3421					0			0			0
3423	4,4′-Diamino diphenyl	0.12			++			+++D			
3424	sulfone				++		++D				
3427					++D						
3431	Sulfadiazine	0.5			0			0			0
3432	and Quinine	0.25			0			0			0
3433					0			0			0
3434					0			0			0
3435					0			0			0
3436	Quinine	0.25			+++		++D				
3437					+++	D					
3438					+++			++++			+++
3439					+++		++++D				
3440					++			++++			+++
3441	Atebrin	0.12			+++	D					
3442					+++		+++D				
3443					+++			D			
3444					++++D						
3446	Plasmochin	0.12	+D								
3447					+D d						
3449			0D								
3452	Diamino diphenyl	0.12			++	D					
3453	sulfone derivative				++	D					
3454					++			++			+++
3455					++			++		D	
3456	*Sulfadiazine	0.5			0						0
3458					+d			0			0

* Drug given seventh day to sixteenth day.
d = damaged exoerythrocytic forms.
+ - ++++ = Intensity of exoerythrocytic forms. D = Died.
Controls shown in Table IV.

TABLE IV.

Effect of Sulfonamides, Diamino diphenyl sulfone, and Plasmochin after the Appearance of Exoerythrocytic Forms. Drug fed in mash from ninth day to fifteenth day.

Chick No.		% of drug in mash	Biopsies on days following inoculation									
			8	9	10	11	12	13	14	15	16	17
3460	Sulfaguanidine	0.5	++			++	+++D					
3461			+			++	D					
3463			++			+++	D					
3469			+			++	D					
3465	Sulfathiazole	0.5	++		+++D							
3466			++			+++	D					
3472			+				+++D					
3480			+			++	D					
3467	Sulfadiazine	0.5	++			++d			+d			0
3468			++			++d			+d			0
3481			+			+++d			++d	D		
3485			+			++d	D					
3470	Sulfapyrazine	0.5	++			++			+d			+d
3473			++			++++D						
3486			+			++d			0			0
3487			+			++d			++d			0
3462	Plasmochin	0.25	0	+		+		+D				
3477			0	+		++		++D				
3493			0	++		++		++++D				
3475	4,4′-Diamino-	0.25	0	++		+++D						
3482	diphenyl		0	+		++		+	D			
3495	sulfone		0	+		++	D					
3459	Controls		0	+		0			+++D			
3476			++			++	D					
3478			++			+++		++++D				
3479			++			++++	D					
3484			0	++		+++	D					
3488			0	+		++		+++D				
3489			+			+++			++++	D		
3490			0	0		++			++D			
3491			0	0		0			+	+D		
3492			++			+++	D					
3494			++			+	D					
3496			+				+++D					

d = Damaged.
+ - ++++ = Intensity of exoerythrocytic forms.
D = Died.

brain capillaries from the seventh to ninth day after inoculation. There is a direct relationship between the number of sporozoites injected and the appearance of these forms as a greater number of sporozoites decreases the time of appearance of exoerythrocytic parasites. Two chicks inoculated with 6,500,000 sporozoites each died on the fifth day with minimal blood infections but with an advanced degree of exoerythrocytic involvement.

If chicks are inoculated with infected red cells, exoerythrocytic forms do not appear until much later, approximately the eighteenth day. Deaths before this interval are usually negative for exoerythrocytic forms. These findings confirm earlier reports based on autopsy examinations. However, by the above described technic it was discovered that an occasional animal inoculated either with sporozoites or trophozoites, will show some exoerythrocytic stages and spontaneously recover. (See Table I.)

Effect of Various Drugs on Exoerythrocytic Forms on Induced Infections. Sulfaguanidine, sulfapyridine, sulfathiazole, sulfadiazine, sulfanilamide and quinine were first tried for their effect on trophozoite-induced infections. The inoculating dose contained 10,000 parasites. Treatment was withheld until exoerythrocytic stages appeared in biopsy specimens, then was given *per os*. Details of the experiment are summarized in Table II. It is shown that of 13 chicks receiving sulfonamide drugs 10 recovered and 3 died. All 3 quinine-treated chicks died. Two of 4 controls recovered although exoerythrocytic stages were demonstrable in all. It was of interest to note that effective drugs could be detected before there was any diminution in the numbers of exoerythrocytic stages. Visible damage recognized by vacuolation, loss of definition, and poor staining was very evident in the stained smears. In the normals which recovered there was a gradual disappearance of the exoerythrocytic stages.

The above experiment was repeated several times, with differences in dosages and variety of compounds. Instead of giving drugs in interrupted doses in some instances they were incorporated in mash. It was found that quinine and atebrin and a wide variety of other compounds were totally without effect. A positive effect with plasmochin was barely demonstrable. The above-mentioned sulfonamides were regularly effective and 4,4′-diamino diphenyl sulfone was also partially effective in destroying the exoerythrocytic stages after they had made their appearance in the brain capillaries.

Effectiveness of Sulfonamides, Sulfones, Quinine, Atebrin, and Plasmochin on Exoerythrocytic Stages in Sporozoite-Induced Infections. Because of the differences in appearance of the exostages depending upon whether sporozoites or trophozoites were used to induce an infection, the experiments were repeated using sporozoites as the infecting agent. As mentioned above, in infections following sporozoite inoculations in routine dosages, exoerythrocytic stages appeared on the seventh to ninth day. In Table III details of drug dosages and results of biopsy examinations are given.

The results of this test showed that sulfadiazine alone or with quinine prevented the appearance of exoerythrocytic forms, although the drugs were withheld until the fifth day after inoculation. Quinine and atebrin were without effect. Plasmochin had a minimal retarding effect.

In order to subject the drugs to a more rigid test, a number of chicks from the same group were selected and therapy was withheld until the appearance of the exoerythrocytic stages. The details and results of this test are furnished in Table IV.

From this experiment it can be seen that sulfadiazine and sulfapyridine destroyed the exoerythrocytic forms even after they had commenced to occlude the brain capillaries. Quinine and atebrin were unable to influence their development, although they were effective on the asexual forms in the blood.

Summary and Conclusions. A method is described for obtaining biopsied brain tissue containing exoerythrocytic stages of *Plasmodium gallinaceum* throughout their course of development in individual chicks. With this method it is feasible to examine individual animals repeatedly without appreciable harm, thus making it a valuable technic for any study on exoerythrocytic schizogony. Earlier

studies made from autopsies have been confirmed. In addition it has been shown that in occasional animals exoerythrocytic stages were encountered which spontaneously disappeared, although this finding is infrequent. From the standpoint of chemotherapy, it has been found that sulfadiazine will prevent the appearance of these forms. Likewise this same drug or sulfapyrazine will effect a cure and cause a disappearance of exoerythrocytic schizonts even after they have made their appearance. Quinine and atebrin have no prophylactic or curative action. Plasmochin has a questionable therapeutic effect. The implications of this study are evident if it is eventually shown that exoerythrocytic schizogony is an integral part of human malarial infections. However, regardless of its place in the life cycle of plasmodial infections, the fact that these forms respond so readily to some of the sulfonamide compounds and yet are totally refractory to quinine or atebrin furnish another lead in the study of malarial chemotherapy.

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Some Chemotherapeutic Properties of Sulfaquinoxaline.

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Following the synthesis of sulfaquinoxaline by Weijlard, Erickson, and Tishler[1] and the report by Seeler *et al.*[2] on the pharmacological properties of this compound, it was thought of interest to determine the *in vitro* and *in vivo* efficacy of this drug against certain bacteria.

In Vitro. Using a modification of Kolmer's method[3] and a strain of *E. coli* grown in a synthetic medium,[4] sulfaquinoxaline was found to be 4 times as effective as sulfanilamide and 32 times less effective than sulfadiazine. As reported for other sulfonamides, sulfaquinoxaline was mainly bacteriostatic and not bactericidal. Thus over a 72-hour incubation period, sulfaquinoxaline, sulfanilamide, and sulfadiazine caused complete inhibition of the test organism in dilutions of 1:64,000, 1:16,000, and 1:2,000,000 respectively. As previously observed with the sulfonamides,[5] sulfaquinoxaline caused definite morphological changes in dilutions far greater than those required to inhibit the growth of the organism.

In Vivo. For the *in vivo* tests, mouse-virulent strains of *Diplococcus pneumoniæ* Type I, *Streptococcus hemolyticus* 1685, *Salmonella schottmülleri,* and *Salmonella aertrycke* were used. Details of maintaining the growth and virulence of these cultures have been described in a previous paper.[6]

The mice were infected by intraperitoneal injections of 0.5 cc of 10^{-4} to 10^{-6} dilutions of a six-hour culture of the above organisms. The amounts of culture were adjusted to equal approximately 10,000-100,000 lethal doses, as determined by titration in mice. All experiments were performed using suspensions of sulfaquinoxaline, sulfadiazine, and sulfathiazole prepared immediately before use by grinding the powder in 10% gum acacia. Treatment with the 3 drugs was given by oral administration immediately following the bacterial inoculation. Subsequent therapy with sulfaquinoxaline was given once every 24 hours, together with 0.5 mg/mouse of vitamin K_1. It has been shown by Seeler *et al.*[2] that sulfaquinoxaline produces a hypoprothrombinemia which can be prevented by feeding

1 Weijlard, J., Erickson, A. E., and Tishler, M., in press.

2 Seeler, A. O., *et al.*, in press.

3 Kolmer, J. A., and Boerner, F., *Approved Laboratory Technic,* 3rd Ed., 1941.

4 Kohn, H. I., and Harris, Jerome S., *J. Pharm. and Exp. Ther.,* 1941, **73**, 344.

5 Lockwood, J., *J. Immunol.,* 1938, **35**, 155.

6 Robinson, H. J., *J. Pharm. and Exp. Ther.,* 1943, **17**, 70.

TABLE I.

Efficacy of Sulfaquinoxaline in Mice Infected with *D. pneumoniæ*. (Oral Therapy.)

Organism: *Diplococcus pneumoniæ* Type I, No. 37.
Age of Culture: 6 hours.
Infection: 0.5 cc of a 10–6 dilution in broth.
Therapy: Sulfaquinoxaline, sulfadiazine, or sulfathiazole given immediately after bacterial inoculation.

No. of mice	Drug	Mg/dose	No. of doses/day	Culture dilution	No. surviving in days														% Survival
					1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
				Therapy: Single daily doses over a 5-day period.																
40	Sulfaquinoxaline*	5	1	10–6	39	30	25	17	12	8	7	5	5	5	5	5	4	4	4	10
80		10	1	”	79	72	60	49	44	35	28	23	19	19	19	19	17	17	17	21.3
80		20	1	”	80	79	75	69	66	54	48	40	39	38	37	37	37	36	36	45
40		40	1	”	40	39	36	35	35	34	29	29	29	29	29	29	29	29	29	72.5
40	Sulfadiazine	5	1	10–6	38	23	12	5	5	4	2	2	2	2	2	2	2	2	2	5
80		10	1	”	79	49	29	25	19	14	8	7	4	4	4	3	3	3	3	3.8
80		20	1	”	80	69	65	41	33	30	28	20	18	18	17	17	17	17	17	21.3
40		40	1	”	39	39	38	28	23	21	19	17	15	15	13	13	12	11	11	27.5
40	Sulfathiazole	10	1	10–6	24	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
40		20	1	”	31	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
				Therapy: Every 6 hrs over a 5-day period.																
10	Sulfaquinoxaline*	1.25	4	10–6	7	5	4	2	1	1	1	0	0	0	0	0	0	0	0	0
10		2.5	4	”	9	8	7	3	2	2	2	2	2	2	2	2	2	2	2	20
10		5.0	4	”	9	6	6	4	3	3	3	3	3	3	3	3	3	3	3	30
10	Sulfadiazine	1.25	4	10–6	8	5	4	3	1	1	0	0	0	0	0	0	0	0	0	0
30		2.5	4	”	30	29	25	23	20	20	10	8	8	8	8	7	7	7	7	23.3
30		5.0	4	”	30	30	30	25	23	22	21	16	15	15	15	15	15	15	15	50
20	Sulfathiazole	2.5	4	10–6	20	13	5	5	3	1	0	0	0	0	0	0	0	0	0	0
20		5.0	4	”	19	18	15	13	9	3	2	0	0	0	0	0	0	0	0	0
				Therapy: 0.																
90	Controls*	—	—	10–6	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
50		—	—	10–7	11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
50		—	—	10–8	17	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2
50		—	—	10–9	31	6	1	1	1	1	1	1	1	1	1	1	1	1	1	2

* Mice also received 0.5 mg vitamin K_1 daily by oral administration.

TABLE II.

Efficacy of Sulfaquinoxaline in Mice Infected with *S. schottmülleri* (Oral Therapy).

Organism: *Salmonella schottmülleri.*
Age of Culture: 6 hours.
Infection: 0.5 cc of a 10–5 culture dilution in 4% mucin.
Therapy: Sulfaquinoxaline, sulfadiazine, or sulfathiazole given immediately after bacterial inoculation.
No. of Mice: 20 to each level.

Drug	Mg/dose	No. of doses/day	Culture dilution	No. surviving in days															% Survival
				1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
Therapy: Single daily doses over a 5-day period.																			
Sulfaquinoxaline*	5	1	10^{-5}	18	17	13	11	10	10	10	10	10	10	10	10	10	10	10	50
	10	1	”	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	100
	20	1	”	20	20	19	18	18	18	18	18	18	18	18	18	18	17	17	85
Sulfadiazine	5	1	10^{-5}	18	18	18	18	17	17	17	17	17	17	17	17	17	17	17	85
	10	1	”	17	16	14	14	14	13	12	12	10	10	10	10	10	10	10	50
	20	1	”	19	19	18	18	18	18	18	18	15	14	12	12	12	12	12	60
Therapy: Every 6 hrs over a 5-day period.																			
”	2.5	4	10^{-5}	20	20	20	19	19	19	19	18	18	18	18	18	18	18	18	90
	5.0	4	”	20	20	19	19	17	14	12	10	10	10	10	10	10	10	10	50
Sulfathiazole	2.5	4	10^{-5}	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	100
	5.0	4	”	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	100
Controls*	—	—	10^{-5}	2	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0
	—	—	10^{-6}	9	2	2	2	2	2	1	1	1	1	0	0	0	0	0	0
	—	—	10^{-7}	13	9	9	9	9	8	7	7	7	7	7	7	7	7	7	35
	—	—	10^{-8}	15	10	9	9	9	9	9	9	9	8	8	8	8	8	8	40

* Mice also received 0.5 mg vitamin K_1 once daily by oral administration.

vitamin K1. For comparison, sulfadiazine and sulfathiazole were given once every 24 hours to one group of mice and every 6 hours to another group. The results of these experiments are given in Tables I and II. For simplicity, only the results at daily intervals with *D. pneumoniæ* and *S. schottmülleri* are given. Essentially the same findings were obtained with *Strep. hemolyticus* and *S. aertrycke*.

When all 3 drugs were given once daily, sulfaquinoxaline was superior to sulfadiazine or sulfathiazole, in that a single daily dose of 40 mg per mouse protected 72.5% of the mice infected with *D. pneumoniæ* Type I. Under the same conditions sulfadiazine and sulfathiazole protected only 27% and 0% respectively. Moreover, single daily doses of sulfaquinoxaline in severe pneumococcal infections compared favorably with multiple daily doses of sulfadiazine or sulfathiazole (Table I). This was found when 20 mg of sulfaquinoxaline was given as a daily dose, as compared with 20 mg of sulfadiazine or sulfathiazole divided into four 6-hourly doses of 5 mg per mouse. Both drugs protected about 50% of the mice.

Tests performed to determine the efficacy of sulfaquinoxaline when given at intervals other than every 24 hours, showed that if treatment was given every 36 or 48 hours, a dose of 40 mg/mouse of sulfaquinoxaline was not sufficient to protect mice from severe infection.

Blood Levels. The striking therapeutic effects obtained with single daily doses of sulfaquinoxaline may be explained by the fact that this drug remains in the blood for long periods of time and consequently, therapeutic blood concentrations can be maintained by infrequent drug administration. Thus, applying the method of Bratton and Marshall,[7] it was found that a concentration of 3 to 7 mg% was still present in the blood of mice 24 hours after the administration of a single dose of 20 mg per 20 g mouse. Even after 72 hours, small quantities of the drug could be detected in the blood. When the interval between treatments was extended beyond 24 hours, the blood concentration of sulfaquinoxaline fell below the effective range, with the resulting death of the animal.

Summary. Sulfaquinoxaline was found to be active against certain bacteria both *in vitro* and *in vivo*. Single daily doses of this drug were more effective than single daily doses of sulfadiazine or sulfathiazole. These findings may be explained by the fact that sulfaquinoxaline remains in the blood for long periods of time.

[7] Bratton, A. E., and Marshall, E. K., Jr., *J. Biol. Chem.*, 1939, **128**, 537.

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The Demonstration of Antibodies in Lymphocytes.*

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The presence in extracts of lymphocytes of normal rabbits of a protein probably identical with the gamma globulin of normal rabbit serum[1] has led to the search for immunologically labeled globulin in lymphocytes of immunized animals. Reticulo-endothelial cells in general[2] and lymphoid tissue in par-

* This investigation has been aided by grants from the Josiah Macy, Jr., Foundation and the Fluid Research Fund of Yale University School of Medicine.

† Alexander Brown Coxe Memorial Fellow.

[1] White, A., and Dougherty, T. F., *Abstracts of Proc. Meetings of Am. Chem. Soc.*, New York, September, 1944.

[2] Sabin, F. R., *J. Exp. Med.*, 1939, **70**, 67.

ticular[3,4] have been suggested as a probable site of antibody production, and we have recently shown[5] that the rate of liberation of antibody from lymphoid tissue is under the control of pituitary-adrenal cortical secretion. The evidence therefore points to lymphocytes as a storehouse of antibody protein. This is now confirmed by the present work which demonstrates the presence of antibodies in lymphocytes.

Experimental. Normal mice of both sexes (NHO strain, Strong), 6 to 8 weeks old at the beginning of the study, received intraperitoneal injections of a 4% solution of fresh, washed sheep erythrocytes on alternate days for 5 weeks. Agglutinin titers were done at weekly intervals on heart blood. Each animal was discarded after being bled once. After 5 weeks of immunization, 2 groups of 5 mice each were bled from the heart and sacrificed. Inguinal, axillary, cervical, and mesenteric lymph nodes, and the thymi were removed and carefully freed from surrounding connective tissues. This lymphoid tissue from each group of 5 mice was pooled and finely minced. The mince was suspended in 3 times its weight of physiological saline, the mixture stirred, and centrifuged in order to free the cells from lymph. This washing was repeated 3 times. Following the last washing, the cells were examined microscopically and found to be essentially normal in appearance. Differential count revealed that at least 90% of the cells were lymphocytes. The cellular mass was lysed by grinding thoroughly with one and one-half times its weight of distilled water. An equal volume of 2% saline was then added and mixing continued for a few minutes. This material was used for analysis.

As controls, the cells from the same lymphoid structures of a group of 5 non-immunized mice of the same strain and age were extracted as described above. For further controls, extracts of muscle tissue and of salivary glands of the immunized mice were

TABLE I.

Agglutinin and Hemolysin Titers in Sera and in Tissue Extracts of Normal and of Immunized Mice.

Material titrated	Mg nitrogen/ml sera or extract			Agglutinin titers			Hemolysin titers		
	Normal mice	Immunized mice I	Immunized mice II	Normal mice	Immunized mice I	Immunized mice II	Normal mice	Immunized mice I	Immunized mice II
Lymphocyte extract	2.26	2.00	2.10	0*	1-2560	1-2560	0	1-3000	1-3000
Serum	7.70	7.39	8.21	0	1-1280	1-1280	0	1-2000	1-2000
First lymphocyte washings	—†	—	—	0	0	0	0	1-10	1-10
Second " "	—	—	—	0	0	0	0	0	0
Third " "	—	—	—	0	0	0	0	0	0
Salivary gland extract	—	—	3.22	—	0	0	—	0	0
Muscle extract	—	—	2.37	—	0	0	—	0	0

* 0 indicates absence of titer in any dilution.
† — indicates determination not done.

[3] McMaster, P. D., and Hudack, S. S., *J. Exp. Med.*, 1935, **61**, 783.

[4] Ehrich, W. E., and Harris, T. N., *J. Exp. Med.*, 1942, **76**, 335.

[5] Dougherty, T. F., White, A., and Chase, J. H., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 28.

obtained in the same manner as those from lymphoid tissue.

Micro-Kjeldahl determinations were made on samples of each extract and on the pooled sera of each group of 5 animals. Agglutinin and hemolysin titers were done on these same extracts and sera. A 2% suspension of fresh, washed sheep erythrocytes was used in all titrations. The titer was read as that dilution of antibody demonstrating complete macroscopic agglutination or hemolysis.

Results. The data are summarized in Table I. The agglutinin and hemolysin titers obtained with the extracts of lymphoid cells from both groups of immunized mice demonstrate that these extracts contained significant quantities of antibody protein. The titers in the extracts of lymphoid cells from immunized mice were approximately eight-fold higher than those in the corresponding sera on the basis of nitrogen contents. The absence of titer in the final washings of the lymphoid cells is proof that the antibody titer in the extracts was derived from cells and not from adherent lymph. Salivary gland or muscle tissue, from the same immunized mice which had yielded antibody-containing lymphoid cells, showed no extractable agglutinins or hemolysins. Also, lymphoid cell extracts and sera from non-immunized mice were negative when tested for antibodies.‡

Discussion. Preliminary attempts were made to extract lysed lymphoid cells with 10% saline, a better solvent for globulins than the 1% salt solution necessary for antibody estimations. However, viscous masses were obtained which contained approximately five milligrams of nitrogen per milliliter. On dilution with water, in order to decrease the salt concentration to 1%, a precipitate of protein formed. After removal of the precipitate, the solutions had nitrogen contents comparable to those found in 1% saline extracts of lymphoid cells, *i.e.*, 2 to 2½ mg nitrogen per milliliter. Therefore, the more dilute saline was directly employed as an extraction medium since the mixtures obtained were easier to manipulate. It is obvious, however, that not all of the cellular globulins, including antibody, were present in the final solutions used for nitrogen and antibody estimations. The titer in the extracts may represent only a fraction of the total antibody of the lymphoid tissue. The considerably higher titers of the lymphocyte extracts, when compared to the corresponding sera on the basis of nitrogen content, indicate a concentration of antibodies within lymphoid cells.

The data substantiate previous suggestions of antibody production in lymph nodes. A significant portion of antibody protein is present in lymphocyte elements of immunized animals. Sites of antibody production and concentration other than lymph nodes may exist, *e.g.*, bone marrow, spleen, liver, and other organs containing high proportions of reticulo-endothelial cells. These have not been examined in the present investigation.

The term "lymphoid cells" has been used in this paper because it is recognized that small numbers of cells other than lymphocytes, designated by the term "lymphoid," exist in the lymph tissue studied. However, the number of non-lymphocyte cells was exceedingly small, as shown by differential count. The antibody nitrogen contributed by reticulum cells, macrophages, or fibroblasts must be insignificant. This is further substantiated by the fact that these cell types are present in the minced salivary gland and muscle tissue of the immunized mice. Extracts of these tissues gave negative titers. The evidence obtained is overwhelmingly in support of the conclusion that the antibody is concentrated chiefly within lymphocytes. The actual production of antibodies by lymphocytes has not been established.

The titer present in the first washings of the minced lymphoid tissue must be derived not only from lymph but also from cells injured by the mincing process. This suggestion is confirmed by the absence of antibody in the subsequent washings. The negative findings with both glandular tissue containing large numbers of cells, and with other tissue from immunized mice, indicate that the antibody in extracts of washed lymphoid cells is present within these cells and is not adsorbed on their surfaces.

The rate of release of certain immune

‡ These extracts and sera were titrated in a one-to-one dilution.

bodies from lymphocytes is under pituitary-adrenal-cortical control.[5] The lymphocyte functions as a carrier of antibody and yields this protein at an increased rate in circumstances of augmented adrenal cortical secretion. The hormonal control of an important lymphocyte function integrates the role of the lymphocyte and the adrenal cortex in maintaining certain normal protective mechanisms of the organism.

Summary. Agglutinin and hemolysin titers have been obtained from extracts of washed cells secured from selected lymph nodes and thymi of mice immunized to sheep erythrocytes. The titers of similar extracts of salivary glands and muscle of the same immunized animals were negative in all dilutions although these extracts were higher in nitrogen content. Per unit of extractable nitrogen, lymphoid tissue had significantly higher agglutinin and hemolysin titers than did the sera of the same animals. It is concluded that antibodies are concentrated in lymphocytes.

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All preliminary manuscripts are indicated by the letter P after the number of the article